Carrier Detection in the Haemophilias

David James Perry

MD
University of Edinburgh
1992

Declaration.

I declare that this work presented here, unless otherwise acknowledged is entirely my own and has not been submitted for publication in any form.

Signed:

David J. Perry

Acknowledgements.

My grateful thanks to Dr. FGH Hill for his advice, encouragement and endless enthusiasm throughout the course of this Thesis.

I am grateful to Dr. Muriel Seaman in the Department of Haematology, Addenbrooke's Hospital, Cambridge and Dr. John Yates in the Department of Genetics for clinical collaboration and the provision of patient samples. I would like to thank Dr. Elizabeth Nacheva for performing the cytogenetic studies, Professor F. Giannelli for Factor IX sequence analysis and Sister Marion Gregory of the Haemophilia Unit of the Birmingham Children's Hospital for her assistance in collecting blood samples.

I am thankful to Professor Robin W. Carrell for allowing me to perform some of this work within his Department.

Finally I must thank my wife, Denise for remaining my wife during the preparation of this Thesis!

I gratefully acknowledge the support of the Wellcome Trust and the Haemophilia Society of Birmingham.

Abstract.

Haemophilia A: 44 families with Haemophilia A have been studied comprising 107 males and 125 females. In 22 families there was only a single isolated affected male whilst in the remaining 22 there was a family history of haemophilia. Of the males 53 were haemophiliacs and of these 37 had severe disease (VIII:C <0.01iu/dl), 7 had moderate disease (VIII:C >0.01 but <0.05iu/dl) and 9 had mild disease (VIII:C >0.05iu/dl). From pedigree analysis 40 women could be established as obligatory carriers, 5 women were considered to be normal and the remaining 80 women were potential carriers. Phenotypic analysis using assays of VIII:C, vWF:Ag, vWF:RCo and the ratios VIII:C/vWF:Ag and VIII:C/vWF:RCo were used to predict carriership and the results compared to those obtained using genotypic analysis with 3 intragenic (Bcl I, Bgl I, Xba I) and 2 extragenic (Taq I, Bgl II) restriction fragment length polymorphisms.
A control group of 31 age-matched normal females and obligatory carriers was used to establish 'cut-off' values for the VIII:C/vWF:Ag and VIII:C/vWF:RCo ratios which correctly classified all of the normal women. Evaluation of this 'cut-off' value in the control group demonstrated the VIII:C/vWF:Ag ratio to be 'superior' to the VIII:C/vWF:RCo ratio correctly classifying 65.2% of the obligate carriers compared to only 21.7% using the VIII:C/vWF:RCo ratio.
Within the study, of the 40 obligate carriers identified by pedigree analysis, coagulation data was available on 37. Using the VIII:C/vWF:Ag ratio, 21/37 (56.8%) were shown to have an abnormal coagulation phenotype and 16 to have a normal phenotype. Phenotypic analysis of the 80 potential carriers identified within the study showed 27 to have an abnormal coagulation phenotype suggesting carriership, 47 to be normal and in 6 no data was available. Genotypic analysis established 23 of the obligate carriers as informative for one or more of the intragenic polymorphisms, 15 as informative for a linked polymorphism and 2 women were non-informative. Of the 80 potential carriers genotypic analysis alone confirmed 7 as carriers, excluded carriership in 31 but in 42 women carrier status could not be established. However using combined phenotypic/genotypic analysis 10 of this latter group were identified as carriers. Overall, therefore, 48/80 (60%) potential carriers could be offered accurate genetic counselling.
In 8 of the 22 families in which there was only a single isolated affected male, RFLP analysis implicated the maternal grandfather as a possible origin for the mutation in 5 cases.
Three families were identified in which a recombination between the DXS15 and Bcl I loci had occurred giving an overall recombination rate of 7.5%. Significant linkage disequilibrium was demonstrated between the Xba I, Bcl I and Bgl I loci.
Prenatal diagnosis using intragenic RFLP's was available to 33/89 women; using linked, RFLP's and fetal blood sampling to a further 39 women and finally to 17 women using fetal blood sampling alone.

Haemophilia B: 5 families with haemophilia B have been studied comprising 11 males and 12 females. Of the males, 4 were haemophiliacs (IX:C >0.01iu/dl) and the remainder normal. Of the 12 females, 3 could be classified as obligate carriers, 8 as potential carriers and 1 as a probable haemophilic female. Although the mean IX:C for the obligatory carriers was lower than that of a control group, there was a considerable overlap of values. Only limited phenotypic data was available but 2 of 3 obligate carriers and 3 of 4 potential carriers had IX:C assays below the lowest value obtained in a control group of 15 normal women. No CRM^+ families were identified.
Three intragenic polymorphisms (Taq I, Xmn I and Dde I) were used for gene tracking studies. Genotypic analysis established 2 potential carriers as carriers and demonstrated informative polymorphisms in 3 obligatory carriers. All 'at-risk' women could be offered prenatal diagnosis using one of the intragenic polymorphisms.
Studies on the single haemophilic female showed her to be an XO/XX^{inv} mosaic and additionally demonstrated a 76Valine - Glycine mutation in one FIX gene.

Adrenoleukodystrophy: Carrier detection, using the FVIII RFLP's was undertaken in a single family with X-linked ALD. The findings confirmed a suspected male as affected and an 'at-risk' female as unlikely to be a carrier. Additionally, a recombination between the DXS15 and DXS52 loci was suspected.

Table of Contents.

List of Abbreviations.

2-ME	2-Mercaptoethanol
2D	2-dimensional
Ab Neut	Antibody neutralisation
ADP	Adenosine diphosphate
AHF	Antihaemophilic factor
$Al(OH)_3$	Aluminium hydroxide
ALK	Adrenoleukodystrophy
APTT	Activated partial thromboplastin time
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
CIE	Crossed immunoelectrophoresis
CLB	Cell lysis buffer
cM	Centimorgan
CPB	Citrate phosphate buffer
CRM	Cross-reacting material
CRM^+	Cross-reacting material positive
CRM^-	Cross-reacting material negative
$CRM^{reduced}$	Cross-reacting material reduced
DGGE	Denaturing gradient gel electrophoresis
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EIA	Electroimmunoassay
ELISA	Enzyme linked immunoadsorbent assay
EPCT	Erythrocyte consumption test
FIA	Fluorimmunoassay

FIX	Factor IX
FV	Factor V
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXI	Factor XI
G6PD	Glucose-6-phosphate dehydrogenase
GpIb	Glycoprotein Ib
GpIIb/IIIa	Glycoprotein IIb/IIIA complex
HMWK	High molecular weight kininogen
IEP	Immunoelectrophoresis
IRMA	Immunoradiometric assay
IX:Ag	Factor IX antigen
IX:C	Functional factor IX coagulant activity
kb	Kilobase
kDa	Kilo-Dalton
Lin Disc Anal	Linear discriminant analysis
Lin Reg Anal	Linear regression analysis
LMP	Low melting point
Log Disc Anal	Logarithmic discriminant analysis
$MgCl_2$	Magnesium chloride
mRNA	Messenger RNA
MW	Molecular weight
NaOAC	Sodium acetate
NaOH	Sodium hydroxide
NIBSC	National Institute for Biological Standards and Control
NLB	Nuclear lysis buffer
OD	Optical density

OLB	Oligo-labelling buffer
OPD	1,2-orthophenylenediamine dihydrochloride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCT	Prothrombin consumption test
PL	Platelet membrane phospholipid
PPP	Platelet poor plasma
PTC	Plasma thromboplastin component
PTT	Partial thromboplastin time
PVP	Polyvinyl pyrollidone
RFLP	Restriction fragment length polymorphisms
RIA	Radioimmunoassay
RIPA	Ristocetin induced platelet aggregation
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sp.Act.	Specific activity
SSC	Trisodium citrate/sodium chloride
SSCP	Single-strand conformation polymorphism
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TF	Tissue factor
TGT	Thromboplastin generation test
Tris	Tris (hydroxymethyl) amino methane
UV	Ultra-violet
VIII:Ag	Factor VIII antigen

VIII:C	Functional factor VIII activity
VIIIR:Ag	Factor VIII-related antigen
vWD	von Willebrand Disease
vWF	von Willebrand factor
vWF:Ag	von Willebrand factor antigen
vWF:RCo	von Willebrand factor ristocetin cofactor activity
WBCT	Whole blood clotting time

Nomenclature for FVIII and FIX is summarised in Chapter 1.

Chapter 1 - Introduction.

Terminology.

To avoid confusion, the terminology used throughout this Thesis, is that recommended by the International Committee on Thrombosis and Haemostasis (ICTH).[1]

The following abbreviations will be used in conjunction with factor VIII:

Attribute	Proposed	Outmoded
Factor VIII		
Protein	VIII	VIII:C
Antigen	VIII:Ag	VIII:Ag
Function	VIII:C	—
von Willebrand Factor		
Protein	vWF	VIIIR:Ag, VIII/vWF, AHF-like Protein
Antigen	vWF:Ag	VIIIR:Ag, AHF-like Antigen
Function	—	**VIIIR:RCo, VIIIR:vWF

** As no test truly reflects *in vitro* vWF activity, no abbreviation is recommended by the ICTH.

FVIII/vWF: designates the factor VIII procoagulant protein/von Willebrand Factor protein complex.

vWF:RCo: will be used to designate ristocetin-cofactor activity, the vWF-related activity required for the aggregation of human platelets induced by the antibiotic ristocetin.

Factor IX.

The following abbreviations will be used in conjunction with factor IX:

Attribute	Abbreviation
Protein	IX
Antigen	IX:Ag
Function	IX:C

1. Introduction.

The past twenty years has seen significant advances in technology and the development of laboratory tests that allow the more accurate diagnosis of Haemophilia A, B and von Willebrand disease. In addition, an explanation of the ways in which these techniques can be used for detecting carriers of Haemophilia A and B together with advances in obstetrics (improved dating of pregnancies by ultrasound scanning, fetoscopy and fetal blood sampling, chorionic villus sampling) has allowed the development of antenatal diagnosis for these disorders. The application of advances in molecular biology to prenatal diagnosis and carrier detection promises to complement this technology further and alleviate some of the current difficulties in carrier detection and antenatal diagnosis.

The introduction to this Thesis on the application of molecular biological techniques to the study of carrier detection in Haemophilia A and B will, therefore, review:

1. Normal coagulation.
2. The development in our understanding of Haemophilia A, B and von Willebrand's disease.
3. The development in technology that now allows a more accurate diagnosis of these disorders.
4. The contribution of molecular biology to our understanding of haemophilia A and haemophilia B.
5. Carrier detection in haemophilia A and B.

1.1. Normal Coagulation.

Normal coagulation occurs as the result of an ordered series of reactions initiated by the contact of blood with the extravascular tissues (Figure 1). Following injury, small blood vessels contribute to the primary haemostatic response by transient vasoconstriction,[2] whilst exposure of subendothelial fibres initiates both platelet activation[3,4] and the intrinsic pathway of coagulation.[5] Platelet adhesion to the exposed subendothelium involves vWF as an anchor,[6,7] is rapidly followed by a change in platelet shape[8] and the subsequent release of numerous platelet constituents from both the α-granules and dense bodies.[9] Activation of the extrinsic pathway of coagulation follows the exposure of FVII to Tissue Factor (TF), a ubiquitous glycoprotein not normally exposed to blood.[10]

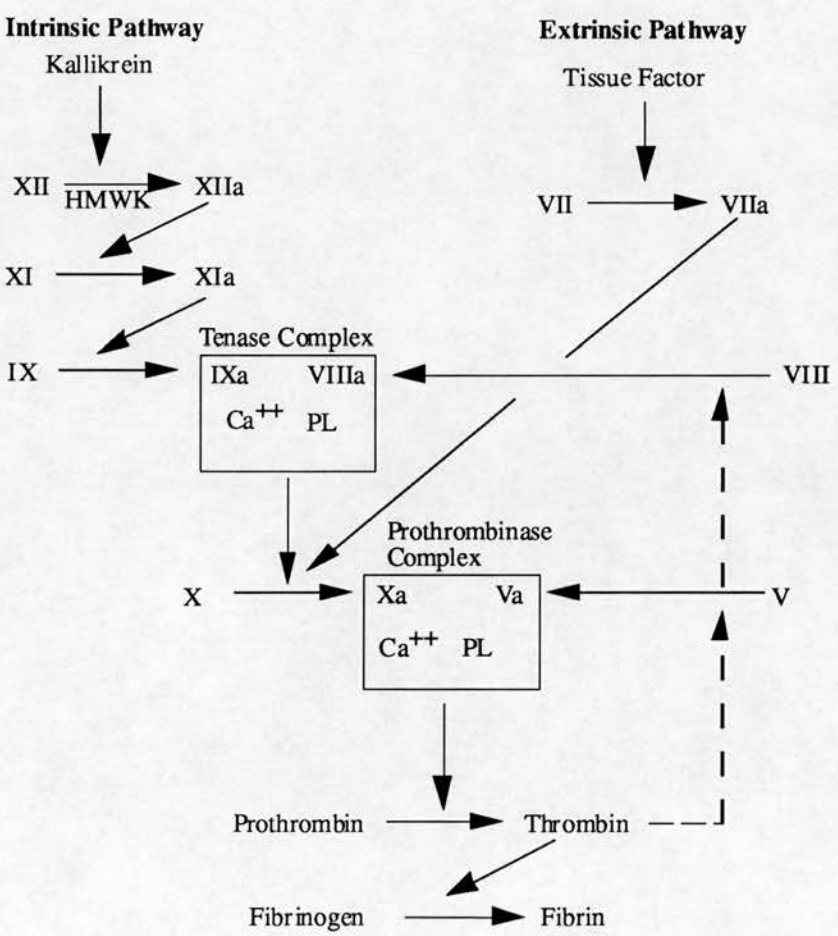


Figure 1. The coagulation cascade illustrating the intrinsic, extrinsic and common pathways of coagulation. HMWK = high molecular weight kininogen; PL = platelet membrane phospholipid; Ca^{2+} = calcium ions.

For many years the coagulation cascade has been divided into the extrinsic and intrinsic pathways[11,12] with a single, final common pathway. The cascade occurs as a series of zymogen to enzyme conversions in which serine proteases generated from a small initial stimulus are enzymatically amplified resulting in a localised, massive burst of thrombin generation which rapidly cleaves fibrinogen to fibrin, thereby stabilising and reinforcing the platelet plug.

Activation of the intrinsic pathway results in the conversion of FXI to FXIa which in turn converts FIX to FIXa. This serine protease binds to FVIIIa and in the presence of phospholipid (from the platelet membrane) and calcium ions, converts FX to FXa.
Factor X can also be activated by an alternative route - the extrinsic pathway. The extrinsic pathway requires tissue factor (TF) for its activation, a protein not normally exposed to the plasma. Exposure of FVII to TF results in the generation of activated FVIIa; the TF-VIIa complex can then convert both inactive FX to the active serine protease FXa and FIX to FIXa.[10]

Finally a complex, prothrombinase, formed from the generation of the serine protease FXa (by either route) and FVa, calcium and phospholipid, converts prothrombin (Factor II) to thrombin which in turn cleaves fibrinogen to fibrin.[13,14]

Whilst deficiencies of all the coagulation proteins have been reported either as a single abnormality or rarely in combination with another factor, only in the last twenty years have the proteins involved in coagulation been purified, cloned and their DNA sequences determined. The commonest of the inherited coagulation disorders are von Willebrand's disease (vWD), Haemophilia A and B (Christmas disease) with approximate incidences of 1/8000,[15] 1/10,000[16] and 1/50,000 respectively.[16]

1.2. Historical Developments In Our Understanding of Haemophilia A, B and von Willebrand's Disease (vWD).

Although the earliest description of haemophilia dates back to Babylonian times,[17] it was not until 1803 that the syndrome of a bleeding tendency occurring in males and transmitted by females was described by Otto[18] and 25 years later Hopff[19] introduced the term 'Haemophilia.' Later in the century, this term began to be restricted to the inherited coagulation disorders having been previously used to describe any bleeding disorder. Bulloch and Fildes (1911)[20] restricted the term haemophilia, on the basis of an extensive literature survey together with their own experience, to describe a 'life-long bleeding tendency occurring only in males, with evidence of similarly affected males in the family with transmission by apparently normal females, and characterised by a prolonged blood clotting time but no other demonstrable abnormality.' In the same year, Addis[21] showed that the long clotting time of haemophilic blood could be corrected by the addition of a small amount of normal plasma. It was not for another 25 years that it became generally accepted that haemophilia was due to the lack of a plasma clotting factor which Patek and Taylor termed 'antihaemophilic globulin.'[22]

The suggestion that haemophilia might be due to more than one defect first arose from the findings of Pavlovsky in 1947[23] that blood from two haemophilic patients was mutually corrective, both *in vitro* and *in vivo*. Similar observations were subsequently made by Schulman and Smith (1952)[24] and in the same year, Aggeler et al[25] and Biggs et al[26] independently described cases which although exactly resembling classical haemophilia clinically and genetically, were shown to be due to a deficiency of another previously unrecognised clotting factor. Aggeler called this new factor 'Plasma Thromboplastin Component' (PTC) and the disease 'PTC deficiency,' whilst Biggs named the bleeding disorder 'Christmas disease' after their first patient and the deficient clotting factor, 'Christmas factor.' Subsequent studies both on the physicochemical and biological properties of these two proteins have shown them to be identical; the internationally agreed nomenclature for this protein is Factor IX.[27]

1.3.1. The Interaction of FVIII and FIX.

Whilst the original Waterfall[11] and Cascade[12] hypotheses of blood coagulation postulated that FIX directly activated FVIII generating FVIIIa which then cleaved FX to its activated form, it is now recognised that this is incorrect and that activated FVIII (FVIIIa) has no intrinsic enzymatic activity and serves only as a cofactor for the serine protease FIXa.[28] Factor IX is a plasma serine protease which circulates as a zymogen and is activated by a specific proteolytic cleavage. This cleavage occurs either by FXIa during activation of the intrinsic pathway or by the TF-VII complex of the extrinsic pathway.

The study of coagulation in reconstituted solution indicates that FIXa has a low but measurable FX activating capacity, which is modestly stimulated by the addition of Ca^{2+}, but the presence of both Ca^{2+} and an appropriate phospholipid, both decreases the K_m for FX and increases the reaction velocity.[29] However, the addition of activated FVIII (FVIIIa) increases the reaction velocity by at least 4000-fold.[30] The increased catalytic activity of FIXa is believed to be due to a direct association of FVIIIa with FIXa on a phospholipid surface but in addition, it is possible that FVIIIa may play a direct role in increasing FX binding to the phospholipid and/or in the modulation of its interaction with FIXa.[31] vWF may also protect FVIIIa from proteolysis by both FXa and activated Protein C[32] although it does not prevent thrombin induced proteolysis of Protein C.[33]

It is clear from the close relationship between FIX and FVIII that a deficiency or functional abnormality of either will result in a failure of activation of the intrinsic pathway.

1.3.2. Definitions of Haemophilia A and B.

Based on our current understanding of the pathophysiology of haemophilia A and B, they may be defined as follows:

Haemophilia A: an X-linked recessive disorder in which there is a lack of procoagulant activity due to either a quantitative or qualitative abnormality of the intrinsic pathway cofactor - FVIII.

Haemophilia B (Christmas disease): an X-linked recessive disorder due to a quantitative or qualitative deficiency of the intrinsic pathway serine protease - FIX.

From the close relationships that exist between FVIII and FIX it can be predicted that a deficiency of either will result in diseases with identical clinical manifestations.

1.3.3. Clinical Manifestations of Haemophilia A and B.

The normal ranges for VIII:C[34] and IX:C[35] in a Caucasian population are 0.50-2.00iu/dl, and the severity of haemophilia, whilst broadly constant within a kindred is closely related to the functional factor VIII or IX activity. The most severely affected cases will have functional factor assay values less that 0.01iu/dl, whilst mildly affected individuals will have values above 0.05-0.10iu/dl. Mild cases of haemophilia may exhibit no problems until their clotting system is stressed eg. dental extraction, surgery. In contrast, severely affected individuals have recurrent haemorrhages into joints, muscles and soft tissues occurring apparently spontaneously or after trivial injury. Repeated bleeds into a joint leads to loss of movement, followed by progressive disorganisation with crippling of the patient. Bleeding into soft tissues may cause life-threatening complications if it occurs within a confined space eg. neck, mouth. Intracranial and intraspinal bleeding are also commoner in haemophiliacs than in the general population

and may be difficult to diagnose in their early stages. Mucous membrane bleeding is a less constant feature of haemophilia but may occasionally occur without any apparent local cause. Epistaxes and haematuria may also occur but are an infrequent cause of bleeding in severely affected haemophiliacs. A major complication of severe haemophilia A and to a lesser extent haemophilia B is the development of inhibitors (antibodies) to the infused FVIII or FIX used to treat the disease.

1.4. Von Willebrand Factor (vWF) and von Willebrand's Disease (vWD).

In 1926, Eric von Willebrand described an Åland Island family with a severe, hereditary bleeding disorder in whom the bleeding time was prolonged but the platelet count, coagulation time and clot retraction were all normal.[36] Evaluation of this family using a 'capillary thrombometer' focused attention on platelet function, for these patients had prolonged 'thrombometer' times. The capillary thrombometer test measured the duration of liquidity when whole blood was pumped back and forth in a capillary tube and since it was normal in haemophilic patients, von Willebrand concluded that the bleeding disorder in these individuals was due to a 'constitutional thrombopathy.'[37]

Over the next twenty years, many patients with 'pseudohaemophilia' as this condition was termed, were described, the diagnosis being based upon a prolonged bleeding time, with a normal platelet count, whole blood clotting time and an autosomal pattern of inheritance. In 1953, a number of groups[38,39,40] reported that vWD was associated with a reduced factor VIII level as well as a prolonged bleeding time. Subsequent work has shown that despite being considered one of the hallmarks of the vWD, the bleeding time may be normal or near-normal in mildly affected cases.[41]

Although the underlying basis of vWD was unclear at this time, it became apparent that the bleeding manifestations of the disease, the reduced FVIII levels and the prolonged

bleeding time were readily treated by infusions of blood, plasma or plasma fractions.[42] These observations indicated that impaired haemostasis in vWD was the result of a plasma factor deficiency and the prolonged bleeding time reflected reduced levels of a plasma 'vascular factor.'[38]

In 1961, Borchgrevink demonstrated a decrease in 'platelet adhesion' *in vivo* in patients with vWD[43] and the *in vitro* analogue, the measurement of the platelet count of blood or platelet rich plasma exposed to glass beads was widely used for many years in the diagnosis of vWD.

A major advance in the understanding of vWF and vWD came with the purification of the FVIII protein and the subsequent development by Zimmerman in 1971, of a specific immunoassay for FVIII using a rabbit anti-FVIII antibody.[44] The measured factor, termed factor VIII-related antigen (VIIIR:Ag) or protein, was shown to be normal in haemophilia A patients but reduced in individuals with vWD. The generally accepted explanation at this time was that haemophilic patients synthesised normal amounts of a dysfunctional factor VIII-like protein but that synthesis of normal FVIII was reduced in vWD. (Subsequent work has shown that this assay measures vWF:Ag rather than VIII:Ag.)

At the same time as immunological techniques were being applied to vWD, a second diagnostic method was developed. For ten years it was known that the antibiotic ristocetin could cause *in vivo* and *in vitro* platelet aggregation and result in severe thrombocytopaenia, but it was not until 1971 that Howard and Firkin demonstrated that ristocetin-induced platelet aggregation (RIPA) was normal in most individuals but not in some patients with vWD.[45] Further study of this defect established a defective or reduced plasma component in vWD and in addition, demonstrated that the platelet defect could be corrected by the addition of normal plasma. In 1973, Weiss[46] modified the system and by employing a fixed concentration of ristocetin, washed normal platelets and dilutions of

control or test plasma, was able to develop a specific quantitative functional assay for this plasma component - FVIII related ristocetin cofactor (vWF:RCo as it was later termed).

By the early 1970's, there was increasing evidence to suggest that FVIII consisted of a dissociable complex. Beginning with the early work of Thelin and Wagner,[47] evidence accumulated that under suitable conditions this macromolecule could be dissociated with the release of active small-molecular weight fragments. In 1973 Weiss and coworkers,[48] Owen et al[49] and Cooper et al[50] demonstrated that at high ionic strength or with alkali halides, detergents or 0.25M Ca^{2+}, the FVIII complex could be dissociated resulting in a large carrier protein and a smaller protein which retained VIII:C activity. In addition, these separate proteins could be recombined to form a large active complex.

It is now known that FVIII circulates as a complex comprising a low molecular weight portion - FVIII which is deficient or abnormal in patients with classical haemophilia A and a high molecular weight portion - von Willebrand factor which constitutes 99% of the complex and which is deficient or abnormal in patients with von Willebrand's disease. Although these two proteins circulate as a complex in plasma, each is encoded by genes on different chromosomes and has entirely different properties (Table 1).

Table 1. Properties of Factor VIII and von Willebrand Factor (vWF).

	Factor VIII	**von Willebrand Factor**
Gene locus	Xq28	Chromosome 12 (12p12-12pter)
Site of synthesis	Hepatocyte	Endothelial cell and megakaryocyte
Plasma concentration	50-150ng/ml	5-10μg/ml
Principal biologic activity	Cofactor in FIX activation	1) Platelet adhesion to endothelium 2) Carrier for FVIII in plasma
Assay		
A. Functional	1-stage and 2-stage FVIII assays; APTT; FXa formation	Bleeding time; platelet agglutination, platelet aggregation eg. ristocetin
B. Immunologic	IRMA; ELISA; immunoblot; inhibitor neutralisation	**Quantitative:** IRMA, ELISA, Laurell electroimmunoassay **Qualitative:** CIE; multimeric analysis: immunoblot
Inheritance	X-linked recessive	Autosomal
Clinical disorder	Haemophilia A	von Willebrand disease

APTT - activated partial thromboplastin time; IRMA - immunoradiometric assay; ELISA - enzyme linked immunoadsorbent assay; CIE - crossed immunoelectrophoresis.

1.4.1. Cloning of von Willebrand Factor (vWF).

In mid-1985, four groups independently reported the construction of partial vWF cDNA clones using mRNA derived from human endothelial cell cultures.[51,52,53,54] These cDNA clones were used to assign the vWF locus to the distal end of the short arm of chromosome 12[51,54] and additionally demonstrated the presence of a pseudogene on chromosome 22.[55,56] In 1986 full-length vWF cDNA's were constructed, sequenced and the predicted amino acid sequence[57,58] shown to agree with the data obtained from amino acid sequencing.[59]

1.4.2. Synthesis of von Willebrand Factor.

The vWF gene consists of at least 50 exons spread over some 200kb of DNA and codes for a vWF mRNA of 8825 or 8830 nucleotides (a discrepancy exists as to the precise site for the initiation of transcription and, therefore, the length of the 5' untranslated region). The 5' untranslated region consists of either 245 or 250 nucleotides, the coding region of 8439 nucleotides and the 3' untranslated region of 136 nucleotides. vWF mRNA encodes a pre-pro-vWF of 2813 amino acids consisting of a 22 residue signal peptide, a long 741 amino acid prosequence and the mature vWF of 2050 amino acids. The pre-pro-vWF amino acid sequence is made up of repeated domains that are internally homologous or of sequence similarity[55,56] (Figure 2). Approximately 90% of the protein can be accounted for by these repeated structures. A number of functional domains on the vWF protein have been mapped and are shown in Figure 2. These include domains involved in the binding to glycoproteins Ib, (GpIb) and IIb/IIIa (GpIIb/IIIa), collagen, heparin and the FVIII binding site.[60]

More recently, a family of proteins has been recognised that have in common regions of high sequence similarity. These common, repeated sequences were first recognised in von Willebrand Factor and were designated A domains. A number of proteins which have in common a variable number of these A domains have now been described.[61] These

include proteins involved in cellular adhesion, immunity, haemostasis and various components of the extracellular matrix and it is feasible that they are related evolutionarily. Some of the members of this family and their possible functions are summarised in Table 2.

Table 2. Some members of the Type A family of proteins, their ligands and possible functions.

Protein	Ligands	Function
vWF	Collagen, GpIb, heparin	Platelet adhesion and activation
C2	C4b	Complement activation
Factor B	C3b	
LFA-1	ICAM-1, ICAM-2	Lymphocyte adhesion
VLA-1/VLA-2	Collagens	Cellular adhesion
CMP	Collagen, proteoglycans	Extracellular matrix assembly
Collagen Type VI	Collagen	Extracellular matrix assembly

LFA - Lymphocyte Function -related Antigen; ICAM - Intracellular Adhesion Molecule; VLA - Very Late Antigen; CMP - Collagen Matrix Protein.

After removal of the signal peptide, pro-vWF subunits dimerise by disulphide bonding involving free cysteine residues located at the C-terminal ends of the molecules[62] (Figure 3). The large prosequence is cleaved from the amino terminus of the pro-subunit which may occur after dimerisation (this prosequence is secreted as a distinct protein[64,65] and corresponds to the previously reported von Willebrand antigen II - vWF:Ag II.[63] vWF:Ag II appears to be essential for intracellular multimer formation.[63,66,67] Pro-vWF dimers appear to act as promoters for the generation of multimers, a process which probably occurs in either the Golgi bodies or a later secretory

pathway. They are assembled into large multimers (ranging in molecular weight (MW) from 800,000 to 20 x 10^6) by the formation of interchain disulphide bonds involving the amino termini of adjacent subunits in a head-to-head configuration (Figure 3).[58]

vWF is synthesised in two sites, the endothelial cell and the megakaryocyte. The vWF produced in endothelial cells is either secreted[68] or stored in the Weibel-Palade bodies for future release.[69] The vascular endothelium is thought to be the site of synthesis of plasma vWF but it is unclear how the complex of vWF and FVIII is formed. Platelet vWF has been located in the granules, cytosol and platelet membrane and various assays of subcellular fractions shows that virtually all vWF is found in the α-granules. Platelet vWF is secreted from the α-granules during the release reaction.[70] The multimeric structure of vWF in platelets is similar to that of plasma although even larger multimers are found. The analysis of the complex multimeric structure of both platelet and plasma vWF[71] forms the basis by which the qualitative variants are classified.[72]

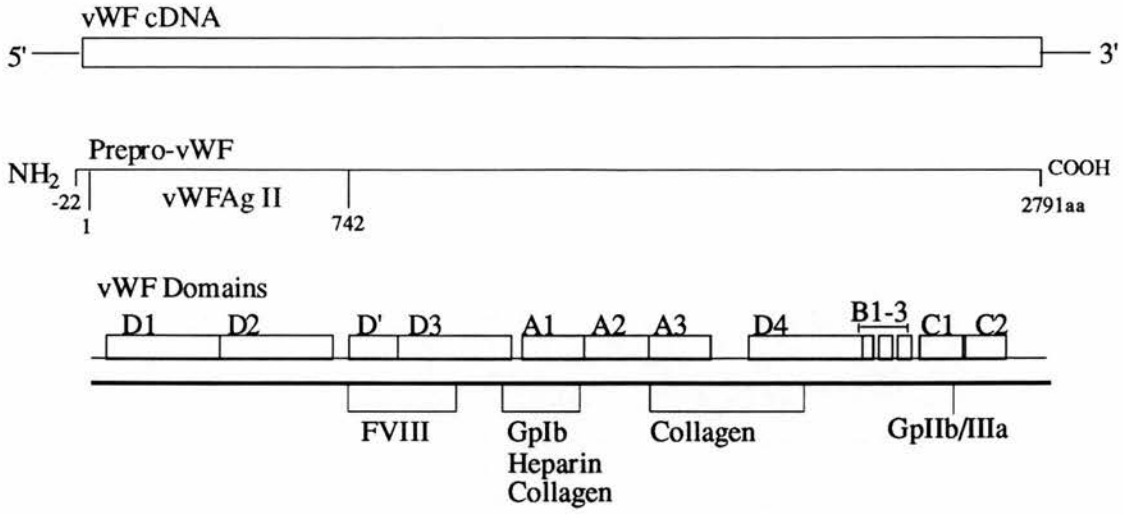


Figure 2. vWF gene transcription and translation products. The functional binding domains are also shown.

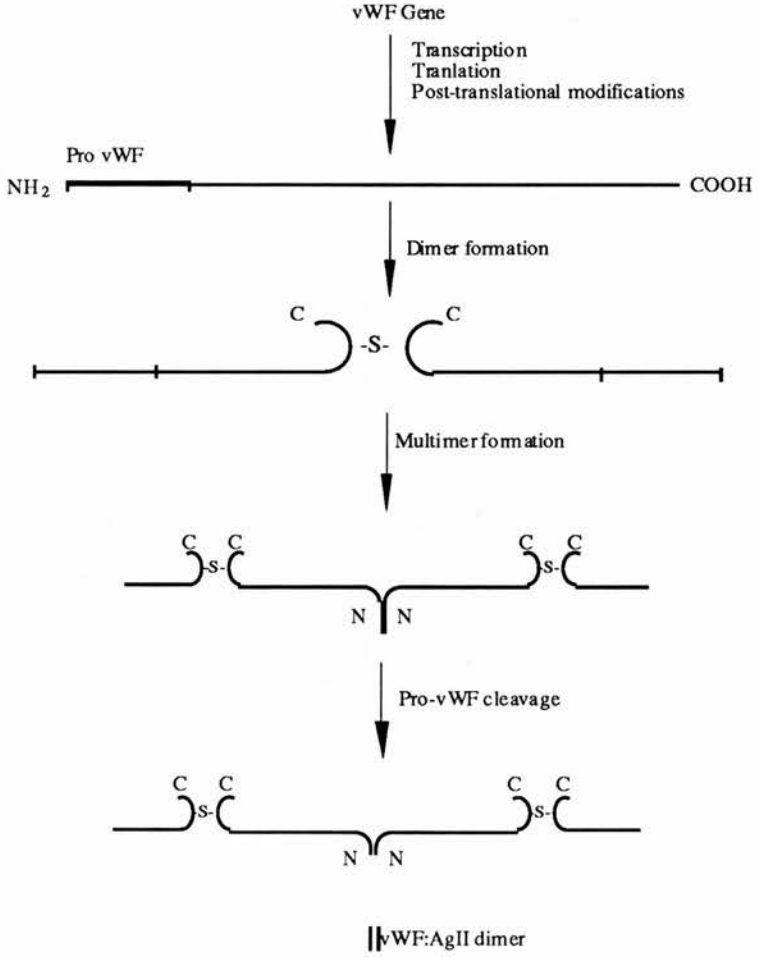


Figure 3. Assembly of the vWF subunit and multimer formation.

1.4.3. Role of von Willebrand Factor.

Although it has no inherent enzymatic activity, vWF is essential for normal coagulation as it promotes haemostasis by anchoring platelets to the vascular endothelium and in addition, serves as a carrier protein for FVIII preventing its proteolytic degradation in plasma.

At shear rates comparable to those that occur *in vivo*, platelets will not adhere to the subendothelium in the absence of functionally active plasma vWF.[73] Two binding sites for vWF have been identified on platelets. The first, associated with the platelet membrane glycoprotein Ib (GpIb) is involved in ristocetin-induced binding of vWF,[74,75,76] whilst the second is associated with the glycoprotein IIb/IIIa complex (GpIIb/IIIa) and binding to this site is induced by both thrombin and ADP.[77,78,79,80] In the subendothelium vWF binds to collagen types I and III[81,82] and to another, as yet undefined, component.[83] vWF may also be involved in platelet-platelet interaction.

vWF acts as a carrier protein for FVIII preventing its proteolytic degradation in plasma[84] and ensures that it is rapidly carried to any site of injury thereby maximising its low concentration in the plasma. The binding site for FVIII lies between residues 1 and 272 on the vWF protein and the actual binding appears to involve calcium ions.[85,86]

1.5. The Development of Quantitative Assays for FVIII, FIX and vWF.

1.5.1. Factors VIII and IX.

In 1893 Wright demonstrated that haemophilic blood had a prolonged clotting time[87] and in 1911 Addis showed that this could be corrected by the addition of a small amount of normal plasma.[88] Brinkhous in 1939 showed that after the coagulation of haemophilic blood was apparently complete it still contained large amounts of unconsumed prothrombin which could then be activated to thrombin by the addition of tissue thromboplastin.[89] The prothrombin consumption test as it was named, though not specific for haemophilia, was more sensitive than the simple clotting time and proved useful in the diagnosis and assessment of the severity of haemophilia. However, such tests were relatively crude, abnormal only in severe disease and failed to differentiate between haemophilia A and B.

In 1951, Merskey[90] described a test in which the calcium clotting time was performed on mixtures (in varying proportions) of normal and haemophilic plasma. In this way a curve relating the clotting time to the dilution of the plasma was obtained and from which, by comparison with an unknown, an estimate of the amount of FVIII present could be obtained. This was the forerunner of a number of tests including the partial thromboplastin time, the activated partial thromboplastin time and the one-stage factor assay.

In 1953 Biggs and Douglas devised a relatively simple test - the thromboplastin generation test[91] in which the formation of 'intrinsic prothrombin activator' ie. prothrombinase was measured. Subsequent modifications of this test have been used to assay both FVIII and FIX - the so-called two-stage factor assay. By substituting the various components of the test with samples obtained from haemophiliacs, Biggs and Douglas were able to demonstrate that haemophilia comprised two separate disorders with

different aetiologies - they called this second disease 'Christmas disease' after their first patient.[26]

FVIII and FIX are now generally measured by one of these two methods - the one-stage or two-stage assay. One-stage assays are widely used and have the advantage of simplicity although the two-stage assay has been shown to be more precise in the measurement of FVIII.[92] A serious drawback to the use of one-stage factor assays is the need for factor deficient plasma. Originally derived from haemophilic plasma the potential infective risks associated with its use make it dangerous although the introduction of heat treated deficient or depleted plasma have reduced these risks.

A number of variables have been shown to affect the accurate assay of VIII:C and IX:C, these include the specimen acquisition and handling, the choice of reagents, standards and buffers, the individual performing the assay and the final analysis of the data.[93,94] This is illustrated by the findings of the College of American Pathologists (CAP) whose survey in 1980 reported that a plasma sample with a target VIII:C activity of 40% when assayed in a number of laboratories by a variety of techniques, resulted in an interlaboratory variation that ranged between 27% and 78%.[95]

Although the introduction of National and International biological standards has considerably improved the standardisation of both VIII:C and IX:C assays some problems in assay reliability still remain.

1.5.2. Von Willebrand Factor.

The introduction in 1970, by Zimmerman and co-workers of a specific immunoassay for FVIII related antigen (or protein) (FVIIIR:Ag)[44] was followed shortly by the demonstration that normal platelets could be aggregated both *in vivo* and *in vitro* by the antibiotic ristocetin.[46,96,97] This led to the finding that both FVIIIR:Ag and ristocetin cofactor activity are present in the blood of haemophiliacs as well as normal individuals,

whilst being absent or reduced in the blood of patients with vWD and led to improvements in the diagnosis of both haemophilia A and vWD. Subsequent modifications to the measurement of ristocetin induced platelet aggregation (RIPA) have allowed the development of a quantitative functional assay for vWF using either platelets[98,99] and more recently of an ELISA assay that reflects function.[100]

With the finding that FVIII comprised two proteins - FVIII and vWF circulating as a covalently linked complex, it was recognised that Zimmerman's original assay measured vWF:Ag (FVIIIR:Ag) rather than VIII:Ag (VIIIC:Ag). However, the assay has proved invaluable both in the investigation of vWD and in the detection of carriers of haemophilia A.

vWF is known to exist in platelets and plasma as a series of multimers of various molecular weight. The diagnosis and classification of the vWD variants is dependent upon the characterisation of these multimers. Two techniques are now widely used for this - crossed-immunoelectrophoresis (CIE)[101] and sodium dodecyl sulphate polyacrylamide (SDS-PAGE) or agarose gel electrophoresis.[102]

In CIE a plasma sample is electrophoresed through a 'first dimension' agarose strip in which no antibody is present. Because vWF is so large the agarose acts as a molecular sieve and separation occurs primarily on the basis of size. Smaller multimers, therefore, migrate more rapidly to the anode than the larger multimers. After the first dimension electrophoresis has been run, a 'second dimension' of agarose gel is poured containing antibody to the vWF:Ag. The plate is turned so that the direction of electrophoresis is perpendicular to the first dimension. The vWF:Ag now migrates into the antibody-containing gel and a precipitate forms, the shape of which reflects the partial separation of multimers produced by electrophoresis in the first dimension. The alteration in the multimeric structure of vWF results in an altered pattern of migration.

A more precise method of resolving the multimeric structure of vWF requires SDS-electrophoresis of either plasma or of platelet lysate using various concentrations of agarose or composite agarose-polyacrylamide gels. Electrophoresis separates the multimers by size, the gels are fixed and reacted with ^{125}I anti-vWF antibodies and the bands subsequently visualised with autoradiography. Multimeric analysis has a superior resolving power compared to CIE and in addition by varying the concentration of agarose in the electrophoresis, various abnormalities of multimeric structure can be highlighted. For example, using a 1% agarose gel each low molecular weight multimer appears as a single band whereas with 2% gels they appear as a triplet with a predominant central band and two satellite bands symmetrically positioned and with even higher resolution gels the multimers migrate as a group of five bands.[103]

Although more recently various other techniques for investigating vWD have become available eg. proteolytic degradation of the vWF subunit, vWF cDNA gene probes, these remain, at present the province of specialised research laboratories.

1.5.2.1. Classification of von Willebrand's Disease.

The classification of von Willebrand's disease is frequently altered as new variants are added but on the basis of vWF:Ag, vWF:RCo and multimeric analysis, they may be categorised as shown in Table 3.

Type I variants are the most frequent form accounting for 60-70% of cases and are due to quantitative defects of vWF with no functional abnormality. All multimers are present but in reduced amounts. Inheritance shows an autosomal dominant pattern and a number of subtypes have been reported.

Type II variants are the qualitative variants of vWF. Inheritance is dependent upon the subtype but usually autosomal dominant. Multimeric analysis shows a lack of the large and intermediate size multimers with variable sub-band abnormalities according to subtype.

Type III is the most severe form of vWD with undetectable vWF. Inheritance is autosomal recessive and affected individuals are usually compound heterozygotes.

Table 3. Classification of von Willebrand's Disease.[72]

vWD Type	Ristocetin aggregation	vWF:Ag	vWF:RCo	Multimeric structure		Pathogenesis
				Plasma	Platelets	
I	Decreased	Decreased	Decreased	All multimers present but reduced in quantity	Variable results in platelets	Reduced synthesis or release of normal vWF
II A	Decreased	Normal or decreased	Decreased or absent	Lack of large and intermediate multimers	Variable according to subtype	Abnormal vWF
C-H	Decreased	Normal or decreased	Decreased or absent	Lack of large and intermediate multimers; abnormal sub-band structure	Variable	Abnormal vWF
B	Increased	Normal or decreased	Decreased or absent	Lack of large and intermediate multimers	Normal	Abnormal vWF - increased affinity for GpIb
III (Severe)	Absent	Very low or absent	Very low or absent	Traces only; some show abnormal multimers	Similar to plasma	Lack of synthesis of normal or abnormal vWF

1.5.3. IX:Ag and the Classification of Haemophilia B.

In 1956 Fantl and colleagues[104] and Roberts and co-workers in 1966[105] used a human antibody to demonstrate that the plasma from some patients with haemophilia B contained material that cross-reacted with a specific FIX antibody. The factor IX:Ag was termed cross-reacting material (CRM). Patients with virtually normal levels of IX:Ag were termed CRM^+. Patients with a proportionate reduction in both factor IX activity and antigen levels were termed CRM^- and those patients with reduced factor IX antigen and a greater, disproproportionate reduction of factor IX activity were called $CRM^{reduced}$ (CRM^r).

Heterogeneity amongst all the categories has been described ie. some patients who have normal IX:Ag levels are very severely affected whilst others are only mildly affected.

A small group of individuals with CRM^+ disease - haemophilia B_m,[106] have been shown to have prolonged ox brain prothrombin times - the hallmark of the disease. A general classification of haemophilia B based upon the measurements of IX:Ag, IX:C and the ox brain prothrombin time is shown in Table 4.[107]

Table 4. A classification for haemophilia B based upon measurements of IX:C, IX:Ag and the ox brain prothrombin time.

CRM status	IX:Ag (iu/dl)	IX:C (iu/dl)	Ox brain prothrombin time	Clinical severity
I CRM^+	Normal	Reduced/absent	Reduced/absent	Severe
CRM^+	Normal	Reduced/absent	Variably increased	Severe
CRM^+	Normal	Reduced/absent	Normal	Severe
CRM^+	Normal	Moderate reduction	Moderate reduction	Mild/moderate
II CRM^r	Reduced	Variable	Normal	Mild/moderate
III CRM^-	Reduced	Matches IX:Ag	Normal	Variable - mild/ Severe

1.5.4. VIII:Ag.

The assays for VIII:Ag are based upon similar principles to those for vWF:Ag although the Laurell technique1[108] is not applicable as no suitable antibody has been found which results in a visible antigen-antibody precipitate. In initial assays the antibody was derived from severely affected haemophiliacs who had developed inhibitors (antibodies) to infused replacement FVIII.[109] More recently alternative approaches using monoclonal antibodies been employed.[110]

The IRMA assay for VIII:Ag reported in 1979 by Peake et al, used an IgG anti-VIII:C antibody arising in a multi-transfused severe haemophiliac.[109] Using this assay they were able to demonstrate that measurements of VIII:C and VIII:Ag correlated well in the majority of normal and haemophilic individuals but in occasional patients normal levels of VIII:Ag were found but reduced or absent VIII:C - CRM^+ haemophilia A. In patients with vWD the levels of VIII:C and VIII:Ag were in good agreement. VIII:Ag was also detectable in serum, cord blood samples and fetal serum and the assay was, therefore, of potential value for both carrier detection and prenatal diagnosis in haemophilia A families.

Despite these initial encouraging reports, there has been little commercial interest in the development of assays for VIII:Ag and currently no widely available commercial kit is obtainable.

1.6. The Contribution of Molecular Biology to our Understanding of Haemophilia A and B.

The contribution of molecular biology to our understanding of the haemophilias has been considerable. With the cloning and sequencing of the two genes, it has become possible to characterise specific mutations within affected individuals, to improve the accuracy and availability of carrier detection to 'at risk' females and more recently to begin to produce sufficient recombinant FVIII and FIX for clinical trials.

1.6.1. The Factor IX Gene.

Historically, FIX was the first of the two proteins to be cloned and sequenced although some 20% of the human[111] and all of the bovine amino acid sequence[112] had been determined by the time the first FIX cDNA clones became available in 1982. The relative ease with which FIX could be purified provided sufficient material to allow amino acid sequencing and the data derived from this proved invaluable in subsequent cloning experiments.

The two groups responsible for cloning the human FIX gene used similar approaches. Kurachi and Davie[113] screened a cDNA library derived from human liver using two different probes. The first probe was derived from baboon liver mRNA enriched for FIX whilst the second comprised a number of synthetic oligonucleotide probes (12), based upon the amino acid sequence Met-Lys-Gly-Lys-Tyr - the sequence having been derived earlier. Using these probes, four positive clones were identified from which a single clone was isolated containing 1,466 base pairs encoding human FIX.

Choo et al[114] used a slightly different approach by using FIX enriched bovine mRNA to synthesise the cDNA isolating positive clones by screening with two sets of synthetic oligonucleotide probes based upon the bovine sequence. The bovine cDNA was then used to probe a human cDNA library, a positive clone was isolated and characterised.
The following year, Jaye et al[115] used a more novel approach by designing a 52-base

synthetic oligonucleotide probe again deduced from the amino sequence of bovine FIX, to screen a human cDNA library and subsequently characterising the positive clones.

In 1984, Anson et al reported the entire mRNA sequence of human FIX,[116] characterised the promoter region and defined the mRNA start, the eight intron-exon boundaries and the mRNA stop point. The following year, Yoshitake published the entire genomic sequence for FIX[119] completing the work started by Kurachi and Davie three years earlier.

The cloning of the FIX gene allowed its localisation to the long arm of the X-chromosome at Xq26-27[118,119,120] centromeric to the Fragile X and FVIII loci. In addition a number of groups identified intragenic polymorphisms which have proved useful in carrier detection studies in haemophilia B families. Cloning has also allowed the development of recombinant FIX although the post-translational modifications necessary to produce functional FIX has made it necessary to employ transformed mammalian cell lines with very low yields (<1mg/litre culture).[121]

The gene for FIX is substantially smaller than that of FVIII but still spans 34kb of genomic DNA,[116,117] although only 4% of the sequence codes for protein. It comprises eight exons which account for 1/12 of the gene, the primary transcript being processed down to a mRNA of 2802 residues (Figure 4) consisting of:

1. A 5' non-translated leader of 29 residues
2. The protein coding sequences
3. A non-translated 3' tail of 1390 residues starting with a double stop translator terminator UAAUAG and ending 15 residues beyond the AAUAAA polyadenylation signal.

The exons of the FIX gene appear to correspond broadly with the domains of the factor IX protein[115] although the catalytic region is divided between two exons.

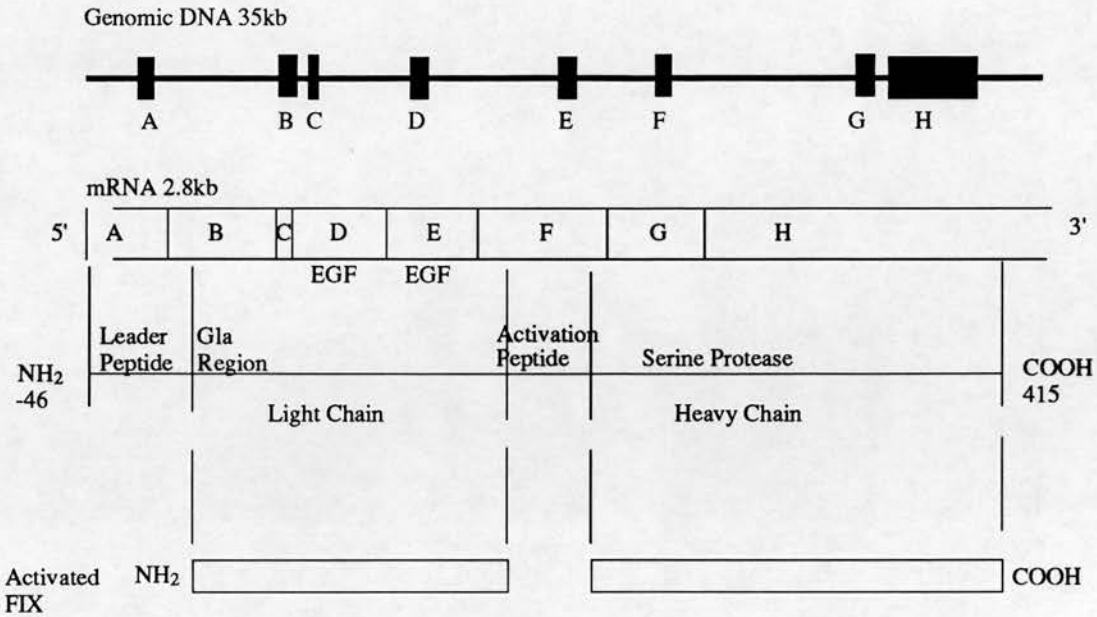


Figure 4. Schematic diagram of the human FIX gene and protein.

The first exon, A, codes for the 5' non-coding region of the mRNA and hydrophobic signal domain of the precursor molecule. Exon B codes for the hydrophilic pro-sequence of the precursor molecule and also for the calcium binding domain (contains 11/12 γ-carboxy glutamyl residues), the so-called 'Gla' region. Exon C, the smallest exon, contains the remaining γ-carboxy glutamyl residue. Exons D and E form the connecting peptide of the protein and have some homology to human epidermal growth factor (EGF). Exon F, encodes the activation peptide whilst the last two exons, G and H code for the serine protease catalytic region of the molecule.

Comparison of the gene structure of FIX with the other vitamin-K-dependent proteins suggests that factors VII, IX, X and Protein C are homologues and presumably represent duplication of the same ancestral gene whilst prothrombin (Factor II) is more distantly related to FIX. [122]

1.6.2. Synthesis of FIX.

FIX, a vitamin K dependent glycoprotein is synthesised exclusively by the liver parenchymal cells resulting in a normal plasma concentration of 3-5μg/ml with a half-life of about 24 hours.[35] Synthesis of FIX is dependent on sufficient amounts of vitamin K in the liver. A vitamin K-dependent carboxylase is responsible for the attachment of the 12 carboxyl-groups to the amino-terminal part of the FIX protein.[123] In the absence of sufficient amounts of vitamin K, acarboxy FIX is produced which may be demonstrated by electrophoresis in the presence of Ca^{2+} ions (migration increased[124]). The levels of FIX are known to be influenced by a number of drugs including oestrogen-containing compounds,[124] anabolic steroids[125,126] and vitamin-K antagonists.[123,127,128]

1.6.3. The Development of FIX Gene Probes.

With the cloning and sequencing of the FIX gene it became possible to characterise the underlying genetic mutations in affected individuals, although until recently such an approach was time consuming and only applicable to small numbers of individuals. More important was the search for polymorphic markers to track mutant genes within a kindred, allowing accurate carrier detection and prenatal diagnosis.

1.6.4. Restriction Fragment Length Polymorphisms (RFLP's).

Polymorphic markers have been used by geneticists for many years to follow the inheritance of genes within a family. If two alleles of a marker can be distinguished by some means then the inheritance of the adjacent linked disease allele can be inferred. Classically the marker gene was a protein eg. glucose-6-phosphate dehydrogenase (G6PD) or Complement 3 (C3) where the different isoforms could be readily distinguished eg. by isoelectric focusing. Such polymorphisms are inherited in a strictly Mendelian manner and allow a mutant gene to be tracked within a family. Such an approach has been used to determine carrier status in haemophilia A using glucose-6-

phosphate dehydrogenase (G6PD) isoenzymes.[129]

Such tracking, however, is dependent upon the marker gene and the disease allele being inherited as a stable unit which is not always so. During the formation of a gamete, a stage in meiosis occurs when DNA is exchanged between the maternal and paternal chromosomes. If this crossing-over occurs between a marker and its linked disease allele then the two will no longer be inherited as a pair and recombination has taken place. The further apart the two markers are on a chromosome the more likely recombination is to occur. To be useful, therefore, a marker should lie within 10 centimorgans (cM) of the disease allele, resulting in an approximate recombination frequency of 10% - a centimorgan is not a measure of physical distance but represents a probability of recombination occurring - 1cM equals a probability of recombination of 1%.[130]

In 1978 Kan and Dozy demonstrated useful polymorphic markers at the DNA level using restriction enzymes.[131] The vast majority of these changes appear to be neutral and many occur in the intronic, non-coding regions of genes. Such polymorphisms may result in the creation or loss of a restriction endonuclease cleavage site and therefore, alter the length of DNA fragments generated using specific enzymes (Figure 5). These so-called restriction fragment length polymorphisms (RFLP's) have proved invaluable in tracking genes in many diseases in whom the underlying genetic defect is unknown eg. haemophilia A and B.

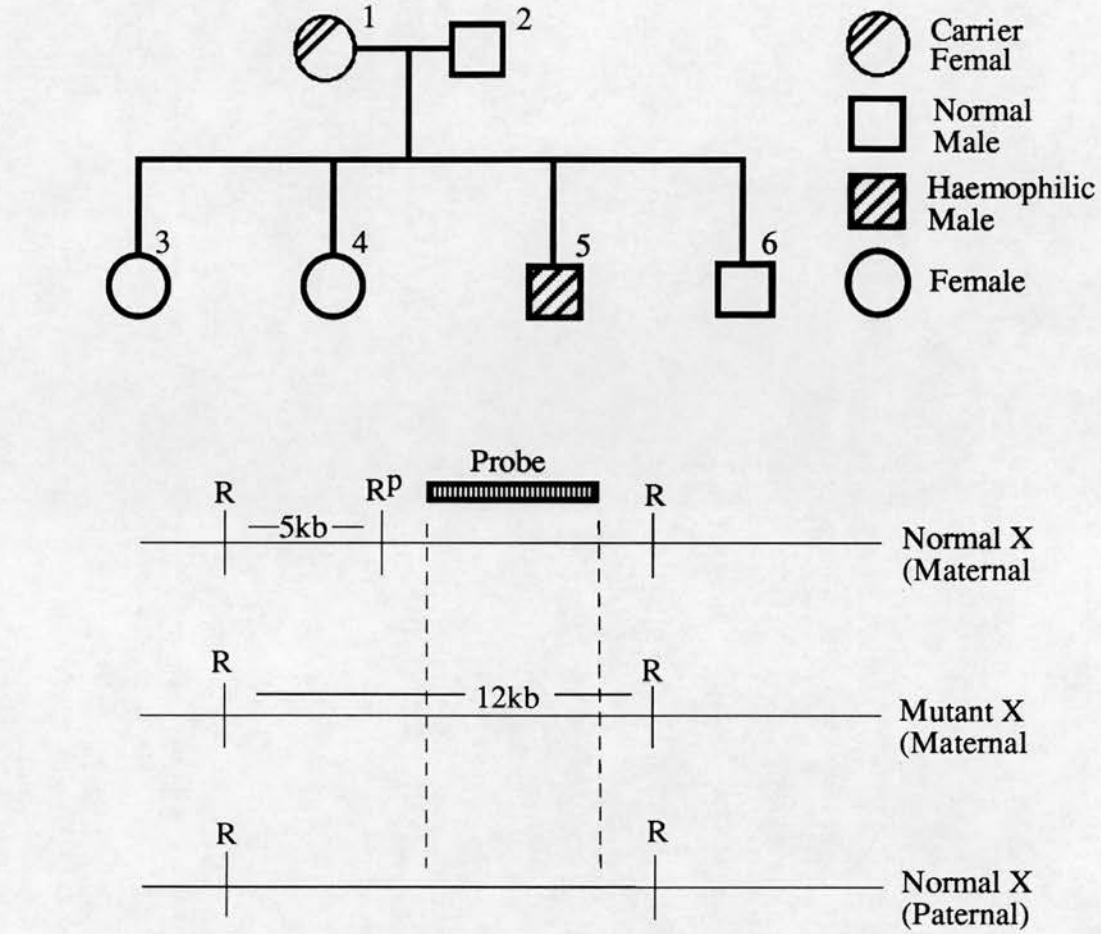


Figure 5. Diagrammatic representation of carrier detection using RFLP analysis. In this kindred the mother (1) is an obligate carrier of an X-linked recessive disease and her two daughters (3 and 4) seek advice to try and establish their carrier status. The probe detects a intragenic polymorphic site (R^P) situated on the X-chromosome. In the absence of the restriction site (R^P), the probe hybridises to a 12kb fragment but when the site is present the probe detects a 7kb fragment. When DNA from each family member is digested and

hybridised with the probe, the mother (1) and daughter (3) are both heterozygous for the polymorphism. The father (2) has a single band of 12kb from his normal gene. An affected son (5) has a single 7kb band which he has inherited from his mother. His sister (4) has only a single band of 12kb, representing a normal 12kb fragment from her father and the abnormal 12kb fragment from her mother and she is, therefore, a carrier. The remaining son (6) has inherited the normal 7kb fragment from his mother and is, therefore, unaffected. The remaining daughter (3) who is heterozygous for the RFLP must have inherited a normal 12kb fragment from her father and a normal 7kb fragment from her mother and she is, therefore, normal.

RFLP's may be divided into two groups depending upon whether they occur within a gene or in regions of DNA closely linked to it:

1. Intragenic polymorphisms
2. Extragenic or linked polymorphisms

1. Intragenic polymorphisms.

Intragenic polymorphisms occur within a gene either within the coding sequences or, more commonly within the non-coding, intronic sequences. Recombination with intragenic markers is a major problem with the analysis of some genes. For example in the case of the dystrophin gene, the chances of intragenic recombination have been estimated at 12%.[132]

2. Extragenic or linked polymorphisms.

Extragenic or linked polymorphisms map close to the gene of interest but are associated with a risk of recombination, the probability of which depends upon the distance between the polymorphic site and the gene.

Although RFLP's have proved invaluable in carrier detection and antenatal diagnosis, their use is associated with many potential problems:

1. A crucial family member may be missing and as a result it is impossible to determine which marker (RFLP) is associated with the disease allele.

2. Non-paternity within a kindred may lead to errors and paternity testing should, therefore, be ascertained whenever paternal RFLP's have to be examined to reach a diagnostic conclusion.

3. Recombination may take place.

4. New mutations may occur.

5. Closely positioned RFLP's exhibit linkage disequilibrium. The finding that certain RFLP's occur more frequently in a population is termed linkage disequilibrium and represents a non-random distribution of alleles at two or more linked loci. Polymorphic protein markers that are in linkage disequilibrium with certain diseases have been known for some time eg. HLA-B27 and ankylosing spondylitis but was first observed in the human genome by Kan and Dozy in 1978 with the ß-globin gene.[131] The rate of change towards equilibrium can be estimated mathematically. If two genetic markers are loosely linked then frequent crossing-over will eventually randomise both forms of one polymorphism with both forms of the other. Eventually all four haplotypes will be present at frequencies proportional to the gene frequencies and the condition of linkage equilibrium will have been reached. However, when the two sites are very close ie. intragenic, the rate of change towards linkage equilibrium due to crossing-over may be very slow and take many thousands of years.

6. The frequency of polymorphisms appears to be lower in the X-chromosome than in other chromosomes.[133]

7. A female may be non-informative ie. homozygous for all available RFLP's.

1.6.4.1. The Detection of Restriction Fragment Length Polymorphisms (RFLP's).

The most commonly used method for detecting RFLP's is known as 'Southern blotting' after its originator Dr. EM Southern[134] (Figure 6). DNA is extracted from, for example, peripheral blood leucocytes, digested with the appropriate restriction enzyme(s) and the fragments separated by agarose gel electrophoresis. The DNA fragments separate in agarose on the basis of their size, very large fragments migrating only slowly whilst smaller fragments move more rapidly. The gel is then soaked in alkali, denaturing the DNA and making it single stranded.

The DNA fragments contained within the matrix of the agarose gel are relatively difficult to probe and are, therefore, transferred to either nitrocellulose or nylon membranes by capillary blotting. The DNA is immobilised on the membrane by either baking (nitrocellulose) or exposure to UV light (nylon).

The hybridisation membranes have a great capacity to non-specifically bind DNA, resulting in a large amount of background radio-activity. This non-specific binding is eliminated by soaking the membrane in non-homologous DNA eg. salmon sperm and then incubating it with the appropriate radio-labelled probe. In the presence of a single-stranded radio-labelled probe, hybridisation occurs only when a complementary sequence is present. Following washing and developing (autoradiography) a signal is observed where complementary sequences are present.

By varying the temperature, pH and ionic strength of the washing buffers (stringency) the degree of mismatch that will be allowed can be varied. At high stringencies, only perfect or near-perfect complementary sequences will hybridise whereas at low stringencies, a greater degree of mismatch between the probe and its complementary sequence is allowed.

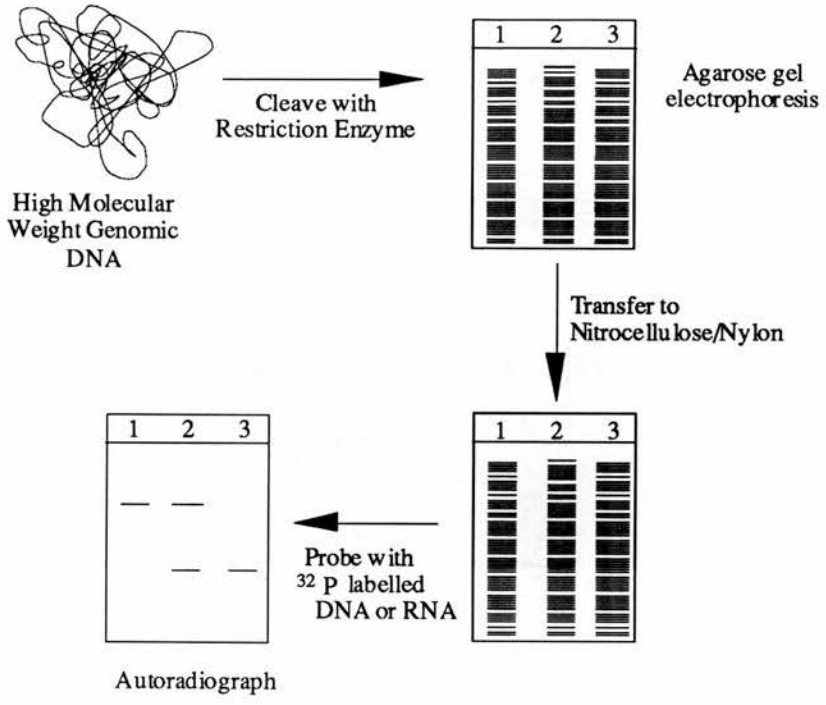


Figure 6. Detection of RFLP's by Southern blotting. Explained in detail in text.

1.6.5. The FIX Gene and RFLP's.

Following the cloning of the FIX gene, Camerino et al in 1984 demonstrated a Taq I polymorphism[120] and in the same year Winship et al established the presence of two further polymorphic markers, Xmn I and Dde I.[135] Figure 7 shows the positions of the currently reported intragenic and flanking RFLP's within or close to FIX gene whilst Tables 5-6 tabulates further information and shows their approximate allelic frequencies in a number of populations.

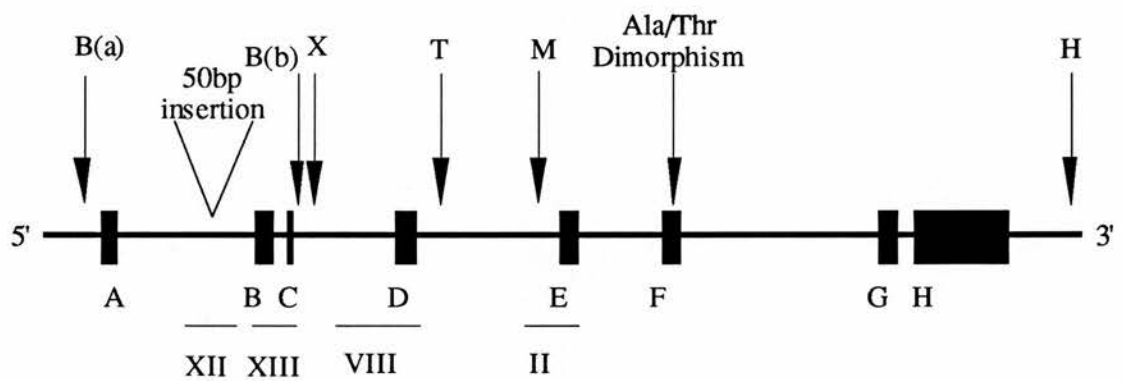


Figure 7. The FIX gene and the location of the currently reported intragenic RFLP's. B(a), B(b) = BamH I sites; X = Xmn I site; T = Taq I site; M = Msp I site; H = Hha I site located 8kb 3′ to the FIX gene. The 50bp insertion detected by Dde I (or Hinf I) and the Alanine/Threonine dimorphism are also included. Roman numerals below the genomic map indicate the positions of the genomic probes used in the detection of the various polymorphisms.

Table 5. Factor IX gene intragenic and extragenic RFLP's.

		Analysis by Southern blotting			Analysis by Polymerase Chain Reaction (PCR) amplification	
Enzyme	**Location**	**Probe**	**Alleles (kb)**	**Reference**	**Fragment sizes**	**Reference**
Taq I	Intron 4	VIII	1.3/1.8	120	124 + 39/163	141
Xmn I	Intron 3	VIII	6.5/11.5	135	154 + 68/222	141
Dde 1	Intron 1	XIII	1.70/1.75	135	317 or /369	141
Msp I	Intron 4	cDNA	3.4+2.4/5.8	136/137	-	
BamH I (a)	5' flanking region	Genomic	23/25	138	216+ 140/356	142
BamH I (b)	Intron 3	VIII	13/15	139	-	
Mnl I[1]	Amino acid residue 148	Oligo-nucleotide	-	140	126+120+159/ 126+279	143
Hha I	3' flanking region	-	-		150 + 80/230	144
Taq I	DXS99	pX58dIIIc	5.9/8.8	150		
Sst I/Sac I	DXS102	pcX38.1	1.65(or 3.9)/11.8	153		

[1]The Alanine/Threonine dimorphism can be detected using several approaches - oligonucleotides probes, PCR followed by digestion with Mnl I and various antibodies.

Table 6. Factor IX RFLP's - racial variation.

White RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Taq I	73	1.3/1.8	0.29/0.71	42	120
Xmn I	72	6.5/11.5	0.29/0.71	42	135
Dde I	74	1.70/1.75	0.24/0.76	37	135
Msp I	74	2.4/5.8	0.20/0.80	32	137
BamH I (a)	96	23/25	0.06//0.94	11	138
BamH I (b)	32	13/15	0/1.0	0	139
Ala/Thr Dimorphism (Mnl I/PCR)	73	0.126+0.120 + 0.159/ 0.126+0.279	0.33/0.67	44	140/145
Hha I	59	0.15+0.080/ 0.230	0.39/0.61	48	144
Taq I (Extragenic)		1.65(or 3.9)/11.8	0.15/0.85	26	150
Sst I (Extragenic)	Not recorded	5.9/8.8	0.43/0.57	49	153

American Blacks RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
					139
Taq I	63	1.3/1.8	0.14/0.86	21	139
Xmn I	60	6.5/11.5	0.12/0.88	21	139
Dde I	40	1.70/1.75	0.64/0.36	46	139
Msp I	49	2.4/5.8	0.61/0.39	48	139
BamH I (a)	21	23/25	0.48/0.52	50	139
BamH I (b)	61	13/15	0.13/0.87	23	139
Ala/Thr Dimorphism (PCR)	91	0.126+0.12 + 0.159/ 0.126+ 0.279	0.11/0.89	20	145
Hha I	91	0.15+0.08/ 0.230	0.57/0.43	49	145

Japanese RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Taq I	81	1.3/1.8	0/1.0	0	146
Xmn I	81	6.5/11.5	0/1.0	0	146
Dde I	81	1.70/1.75	0/1.0	0	146
Msp I	81	2.4/5.8	0/1.0	0	146
Sst I (Sac I)	78	5.9/8.8	0.48/0.52	50	151

Table 6. continued

Chinese RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Taq I	68	1.3/1.8	0.03/0.97	6	147
Xmn I	56	6.5/11.5	0.04/0.96	8	147
BamH I (a)	67	23/25	0/1.0	0	145
Ala/Thr Dimorphism (PCR)	68	0.126+0.12 + 0.159/ 0.126+ 0.279	0.03/0.97	6	145
Hha I	68	0.15+0.08/ 0.230	0.18/0.82	30	145

Asian RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Taq I	80	1.3/1.8	0.09/0.91	16	147
Xmn I	78	6.5/11.5	0.06/0.94	11	147
BamH I (a)		23/25	0/1.0	0	148
Ala/Thr Dimorphism (PCR)	80	0.126+0.12 + 0.159/ 0.126+ 0.279	0.04/0.96	8	145
Hha I	80	0.15+0.08/ 0.230	0.18/0.82	30	145

Malay RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Taq I	69	1.3/1.8	0.01/0.99	2	145
Xmn I	69	6.5/11.5	0/1.0	0	145
BamH I (a)	67	23/25	0/1.0	0	145
Ala/Thr Dimorphism (PCR)	67	0.126+0.12 + 0.159/ 0.126+ 0.279	0.03/0.97	6	145
Hha I	66	0.15+0.08/ 0.230	0.06/0.94	11	145

Maori RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Taq I	72	1.3/1.8	0.07/0.93	13	152
Xmn I	120	6.5/11.5	0.008/0.992	1.6	152

Polynesian RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Taq I	72	1.3/1.8	0/1.0	0	152
Xmn I	116	6.5/11.5	0/1.0	0	152

Heterozygosity - predicted from allelic frequencies (2pq x 100: where p and q represent the frequencies of the two alleles.

1.6.5.1. Intragenic Polymorphisms.

The following intragenic polymorphisms have been used for RFLP studies in haemophilia B:

1. Taq I: situated 3' to exon D and detected by either genomic probe VIII or the FIX cDNA. It detects a two allele system with bands of 1.8kb or 1.3kb.

2. Xmn I: situated 3' of exon C, this polymorphism is detected by the same probes as the Taq I polymorphism and again is a two allele system generating bands of 11.5kb and 6.5kb. However, the two polymorphisms are in strong linkage disequilibrium and little additional information is obtained by the use of the Xmn I polymorphism although occasional families informative for only the latter have been reported.

3. Dde I: situated 5' of exon B and detected by genomic probe XIII or the FIX cDNA probe. This detects a two allele system with polymorphic bands of 1.75kb and 1.70kb. The actual polymorphism represents a small 50bp insert situated in intron 1 of the FIX gene at residues 5505-5554 and which alters the size of the fragments generated by digestion with Dde I. The same polymorphism is also detected with Hinf I but produces fragments of 0.70kb and 0.75kb.

4. Msp I: situated within 10kb of the Taq I site, the polymorphism is detected by either the FIX cDNA probe or a genomic probe and again is a two allele system with bands of 5.8kb and 2.4kb.

5. Alanine/Threonine dimorphism: situated at amino acid 148 in exon F this polymorphism results in the substitution of an alanine for the more common threonine residue. This dimorphism has been detected using a number of approaches including antibodies,[149] oligonucleotide probes[140] and PCR.[143]

6. BamH I (b): Situated 5' to the Xmn I site in intron 3 and detected using the FIX genomic probe - Probe VIII. This again is a two allele system with bands of 15kb and 13kb.

Unfortunately, these intragenic RFLP's are clustered within the FIX gene and as a result their overall usefulness is reduced due to linkage disequilibrium.

1.6.5.2. Linked or Extragenic RFLP's.

A number of linked RFLP's have proved useful in carrier detection studies in haemophilia B.

1. BamH I (a): situated in the 5' flanking sequence of the FIX gene and detected by genomic probe VIII. This is a two allele system with polymorphic fragments of 23kb and 25kb. This RFLP is present in Caucasians but from the allelic frequencies only 11% of females would be expected to be informative whereas in American Blacks the predicted heterozygosity rate is 50%.

2. Hha I: located 8kb 3' to the factor IX gene and detected by PCR. Under normal conditions, the restriction endonuclease Hha I will not cleave at sites where the cytosine residue is methylated. The amplification of specific regions of DNA results in large amounts of non-methylated DNA which can then be cleaved by Hha I permitting its use in the detection of a highly informative polymorphism. Although extragenic the greatest distance between this marker and any potential mutation (assuming all mutations occur within the coding regions or promoter sequences) is 43kb and therefore, the chances of a recombination event occurring are small.

3. Taq I: this polymorphism is detected using the probe pCX38.1 at the DXS102 locus centromeric to the FIX gene.[150] It generates bands of 1.65kb (or 3.9kb) and 11.8kb.

The precise recombination frequency between this extragenic polymorphic marker and the FIX gene is unclear.

4. Sst I/Sac I: this polymorphism is detected using the probe pX58dIIIc at the DXS99 locus which maps telomeric to the FIX gene.[155] It generates bands of 5.9kb or 8.8kb. Again the precise recombination frequency between this extragenic polymorphic marker and the FIX gene is unclear although at least one recombination has been reported.[154]

Summary.

Striking racial differences exist in the prevalence of the FIX polymorphic sites (Table 6). People of Asian and Oriental ancestry show a low frequency of heterozygosity for the Taq I and Xmn I polymorphisms but an increased frequency of heterozygosity for the Hha I polymorphism and in the Japanese for the linked SstI/Sac I polymorphism.
The linked Bgl I has proved to an extremely useful marker in American Blacks where the predicted heterozygosity rate is 50% compared to only 11% in Caucasians.

Overall, the four intragenic RFLP's (Taq I, Xmn I, Dde I, BamH I) and the Alanine/Threonine dimorphism) will be informative in approximately 79% of individuals. By using the data derived from analysis of the Hha I polymorphism the number of informative individuals increases to 89%.[144] It is possible to increase this figure further by using either the extragenic Taq I and/or Sac I/Sst I RFLP's. However, the risks of recombination leading to errors in diagnosis make such an approach unattractive. Improvements in the detection of kindred-specific mutations allowing more accurate carrier detection studies, make the use of these 'distant' linked, extragenic polymorphisms unnecessary.

1.6.6. Kindred specific defects.

Haemophilia B patients may be divided into CRM^+ and CRM^-. The former might be expected to have point mutations in regions of the gene coding for the FIX protein whilst in the latter mutations and deletions critical for the biosynthesis of the mRNA or of the protein may be more common. Approximately 1/3 of cases of haemophilia B are due to a dysfunctional protein and direct sequencing of such variants led to the identification of the first mutant $FIX_{Chapel\ Hill}$ (145 Arg-His) in 1978.[156,157]

Mutations and small deletions/insertions within the FIX gene have recently been comprehensively reviewed by Giannelli et al.[158] Of 216 known mutations, there were 114 unique molecular events the remainder being repeats. The majority of these mutations occurred at CpG dinucleotides, a known 'hotspot' for mutation in the factor IX gene[159] and in the human genome.[160,161] Such mutations arise because the cytosine residue in the CpG sequence is readily methylated generating 5'-methylcytosine. This can spontaneously deaminate to form thymine, resulting in a cytosine to thymine (C-T) mutation.

1.6.6.1. Haemophilia B and the development of inhibitors.

Although a clear association has been shown to exist between the development of inhibitors to infused FIX and deletions within the gene,[162] a number of cases have been reported in which the gene appears normal (by Southern blotting techniques) in the presence of high titre antibodies.[163] Presumably in these cases, small point mutations, deletions/insertions are present. Table 7 shows a number of the reported deletions within the FIX gene and whether they are associated with the development of inhibitors. These are more comprehensively reviewed by Giannelli et al.[158]

The finding that not all deletions are associated with the formation of inhibitors suggests that other factors must be involved in the immune response to therapeutic factor IX. Highly polymorphic immune response genes within the HLA locus in Man are known to

be involved in the presentation of antigens by macrophages and other accessory cells to T lymphocytes as part of the process by which the proliferation of a specific B cell occurs producing antibody. It is possible that individual variations in this system may affect the response to a foreign antigen eg. factor IX replacement and therefore, the formation of antibodies.

Table 7. Deletions and insertions within the FIX gene.

Mutation	**Size**	**Defect**	**Inhibitors**
Manchester I[162,164]	>250kb	Complete	+
Manchester II[162,164]	>250kb	Complete	+
London 1[162,165]	27kb	Exons F,G,H	+
Jersey 1[163]	>114kb	Complete	+
Boston [163]	>114kb	Complete	+
Seattle[166]	11.6kb	Exons E,F	+
Strasbourg[167]	5.0kb	Exon D	-
Chicago[163]	5.0kb 9-29kb	Exons E,G,H	+
Pisa[168]	>35kb	Complete	+
Bari[154]	>3kb	Exons A-H	+
Unnamed[169]	NR	At least exon D	+
" 170	NR	Complete	+
" 170	NR	Complete	+
" 170	NR	Complete	+
" 171	>150kb	Complete	+
" 172	NR	Exons A,B,C	+
" 173	NR	At least exon H	+
Nara 1[174]	>33kb		+
Nara 2[174]	>33kb		+
El Salvador[175]	6kb Insertion	Exon D/intron D	-

NR = not reported.

1.6.7. Haemophilia B Leyden.

An interesting subgroup of unrelated haemophilia B patients are characterised by a similarly altered developmental expression of FIX - the so-called haemophilia B Leyden phenotype, characterised by both low IX:Ag and IX:C before the age of 15 years but with a gradual increase in the parameters after puberty.[176] A number of such cases have been characterised and shown to be due to mutations in the promoter sequence of the FIX gene.[177,178] This has led to the hypothesis that this variant FIX results from a promoter mutation and that sequence elements very close to the putative start site of the transcription are crucial for constitutive FIX transcription. In addition, the results suggest that single-point mutations may suffice to switch from constitutive to hormone-dependent gene expression.

1.7. The Contribution of Molecular Biology to our Understanding of Haemophilia A.

1.7.1. Structure of the FVIII Gene.

It is now recognised that the early preparations of FVIII were purifications of the FVIII/vWF complex and only with the introduction of methods that have allowed the selective purification of the FVIII protein did cloning of the gene become a possibility. This was achieved in 1984 by two Biotechnology companies, Genentech Inc.[179] and Genetics Institute Inc.[180] and represented a remarkable achievement.

Purification of homogeneous FVIII, free of vWF was the necessary preliminary to biochemical characterisation. Tuddenham and his co-workers were able to produce a small amount of highly purified human FVIII protein permitting limited amino acid sequencing. Using this purified protein, Genentech Inc. isolated and sequenced a nonapeptide and from this data, designed a 36-base synthetic oligonucleotide probe based on known codon preferences. This was then used to probe a human genomic library derived from an individual with 4X chromosomes (karyotype 49,XXXXY). The initial clones obtained spanned 28kb of genomic DNA but were subsequently expanded to contain 200kb of DNA encompassing the entire FVIII gene.

The approach taken by Genetics Institute differed slightly. Microsequencing of highly purified porcine factor FVIII yielded sufficient amino acid sequence data to construct oligonucleotide probes which were then used to screen a porcine genomic DNA library. A positive clone was isolated, used to probe a human genomic library and a single recombinant phage of 16kb isolated. Sequencing of the two clones revealed approximate 80% homology and indicated that the human clone represented part of the human FVIII gene. Using fragments of the human clones as probes to screen a human lymphoblastoid cell line (karyotype 49,XXXXY) a number of overlapping clones covering some 200kb of DNA were isolated and shown to contain the whole of the human FVIII gene. The

isolation of genomic and cDNA clones allowed the complete amino acid sequence to be derived.

The gene for FVIII is situated at the tip of the long arm of the X chromosome (Xq28)[181] and comprises 186kb of DNA (0.1% of the human X-chromosome) spread over 26 exons (ranging in size from 69-3106bp) and 25 introns, the six largest of which each contain over 14kb of DNA.[179] This codes for a mRNA of 9,029bp and a precursor polypeptide of 2351 amino acids (Figure 8). The complete nucleotide sequence of the coding regions, the promoter elements, and the intron-exon boundaries have been determined and the amino acid sequence of the protein has been deduced.[179,180,182]

Analysis of the mRNA shows a probable 5' start site at position -170 which is preceded in the genomic sequence by the sequence GATAAA that conforms with sequences required for precise initiation of transcription.[179] The 5' untranslated region is followed by a single, continuous open reading frame coding for the polypeptide protein of 2351 amino acids comprising a 19-amino acid hydrophobic signal peptide which is removed during secretion of the mature protein and the mature protein of 2332 residues. The 3' untranslated region is 1805 residues long and contains the AATAAA and CATTG polyadenylation signals starting 19 bases upstream from the poly(A)$^+$ tail.

1.7.2. Amino Acid Homology Studies.

Computer analysis of the predicted amino acid sequence of FVIII reveals a complex protein with internal homology - Figure 8.

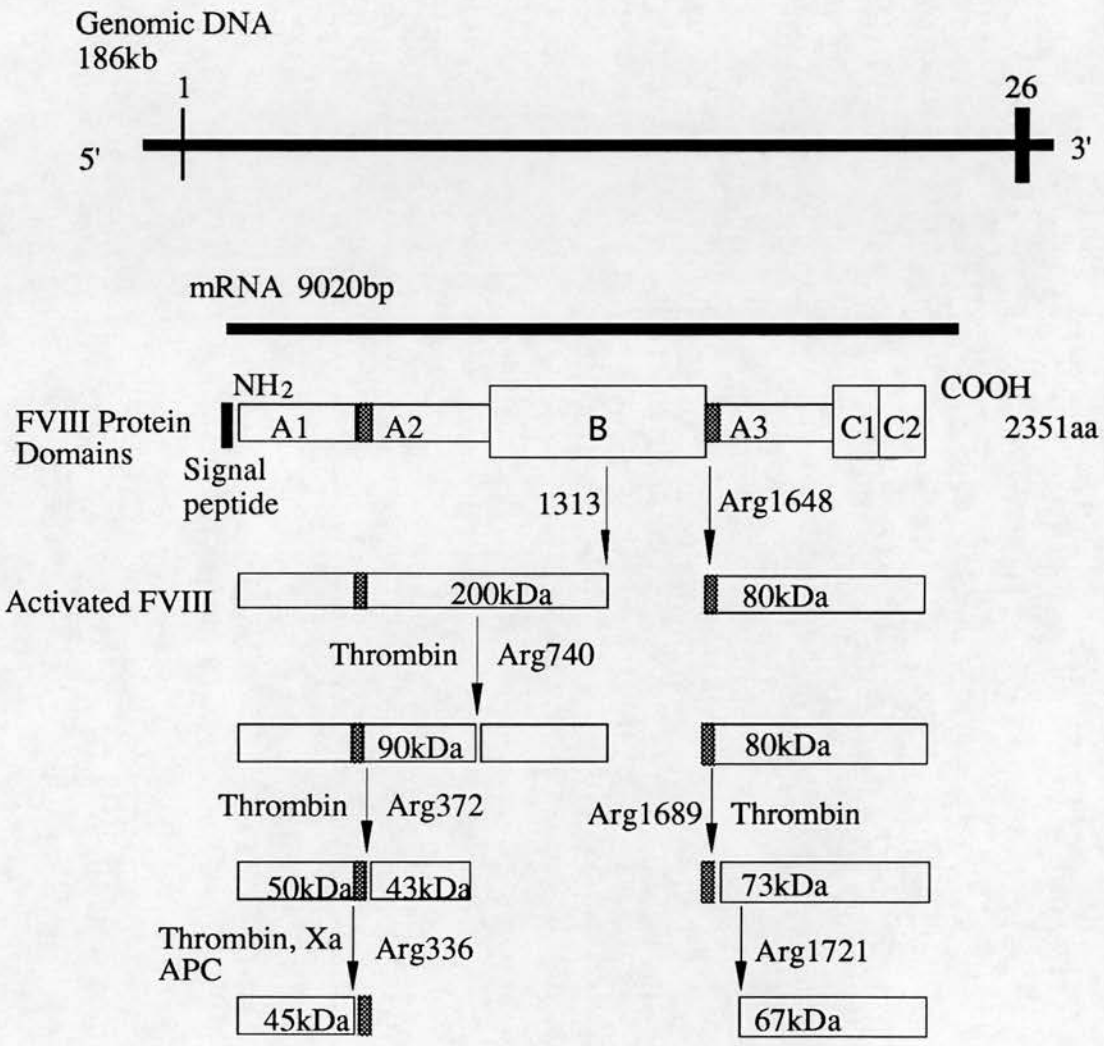


Figure 8. Domain structure and processing of FVIII. The hatched areas represent regions with a high density of acidic amino acids. The thrombin, activated Protein C (APC) and factor Xa cleavage sites are shown.

There are 3 replicated 'A' domains, a single 'B' domain and 2 'C' domains:[182,183]

1. Triplicated A domain:

A1: residues 1-329

A2: residues 380-711

A3: residues 1649-2019

The 'A' domain repeats consist of approximately 350 amino acids with 30% amino acid homology between repeats. The third A domain is found at the amino acid terminus of the light chain following a short segment of 41 predominantly acidic amino acids and it is this region which is thought to contain the binding site for vWF.

2. Duplicated C domain:

C1: residues 2020-2172

C2: residues 2173-2332

The C domain occurs as a tandem repeat at the carboxyl terminus of the molecule and each contains approximately 150 amino acids with 37% amino acid homology.

3. Single B domain:

B: residues 712-1648

The single B domain is coded for entirely by exon 14, as are parts of the A2 and A3 repeats and is presumed to be an ancient insertion.

The mature polypeptide, therefore, has the structure: A_1-A_2-B-A_3-C_1-C_2. The deduced sequence of FVIII contains regions of homology with the known amino acid sequences of bovine factor V and human caeruloplasmin,[180,182] supporting the suggestion that these three proteins are related by evolution. The significance of the repeating homologous units in these three proteins is unclear but may reflect duplications and triplications of some smaller ancestral gene.

1.7.3. Synthesis and Release of FVIII.

Early studies undertaken to identify the source of FVIII production included transplantation studies in which normal organs were transplanted into haemophilic animals.[184] These studies indicated that the liver was the probable source of FVIII production but it was not until the gene was cloned that this was confirmed. Wion and colleagues[185] using FVIII gene probes identified FVIII mRNA in liver, spleen, kidney and lymphocytes and fractionation of liver revealed mRNA in the hepatocyte fraction but not the sinusoidal fraction, indicating the actual hepatocyte as the source of FVIII production rather than other cells within the liver. Endothelial cells which synthesise vWF do not produce FVIII although they may provide a temporary storage site.[186] The control of FVIII synthesis is poorly understood. It is known that hormonal influences can increase the plasma level of FVIII and that vasopressin and its analogues can be used both for diagnostic purposes[187] and in the treatment of mild haemophilia.[188,189] Whether vasopressin acts at the levels of transcription, translation or storage is unclear but the rapidity of its response suggests the latter.

1.7.4. Processing of FVIII.

FVIII serves as a cofactor to FIXa in the activation of FX in the presence of calcium ions and phospholipid from platelets. The rate of FXa production is greatly increased by FVIII whose catalytic efficiency is, in turn, greatly increased by thrombin. Activation and subsequent inactivation of FVIII by thrombin are associated with a series of polypeptide cleavages (Figure 8).

FVIII consists of a heterodimer processed from the larger precursor polypeptide and is stabilised in plasma by complexing with von Willebrand factor. The activation of FVIII coincides with proteolysis of both the heavy and light chains. Cleavage within the heavy chain after Arginine 740 generates a 90kDa polypeptide that is subsequently cleaved after Arginine 372 to generate polypeptides of 50 and 43kDa. Concomitantly the 80kDa light

chain is cleaved after Arginine 1689 to generate a 73kDa polypeptide. Each thrombin cleavage site is bordered by acidic residues and presumably these act as thrombin binding domains. Factor VIII can be inactivated by activated Protein C through proteolytic cleavage within the heavy chain at Arginine 336. Factor Xa and thrombin can also inactivate FVIII by cleavage at Arginine 336.

1.7.5. The Development of FVIII Gene Probes.

Following cloning and sequencing of the FVIII gene in 1984, carrier detection and antenatal diagnosis has involved both RFLP analysis and the detection of kindred specific mutations. However, the much of the work involving carrier detection has, until very recently, made use of RFLP analysis - the size of the FVIII gene making the detection of specific mutations, except in certain individuals, an immense task.

1.7.6. Restriction Fragment Length Polymorphisms and the FVIII Gene.

Despite the size of the FVIII gene, the frequency of polymorphisms appears, as elsewhere on the X-chromosome to be low. In 1984, Harper et al[190] identified a Bgl II polymorphism adjacent to the DNA probe DX13 (DXS15) situated some 5cM from the FVIII gene but tightly linked to it and in the following year Oberlé et al[191] reported a Taq I/Msp I polymorphism associated with the probe ST14 (DXS52), again adjacent to but tightly linked to the FVIII gene. Although both of these polymorphisms have proven extremely useful for in carrier detection and antenatal diagnosis in haemophilia A, their use is associated with a 5% risk of recombination.[192] Cloning of the FVIII gene allowed the search for intragenic polymorphisms and in 1985 Gitschier et al[193] reported a Bcl I site situated in intron 18. In the same year a Bgl I polymorphism was identified[194] and the following year an Xba I site recognised.[195] Tables 8/9 show the currently reported FVIII intragenic and extragenic polymorphisms and tabulates their allelic frequencies in a number of populations. Figure 9 maps their position within the FVIII gene.

Table 8. Factor VIII gene intragenic and extragenic RFLP's.

	Analysis by Southern blotting				Analysis by Polymerase Chain Reaction (PCR) amplification	
Enzyme	**Location**	**Probe**	**Alleles (kb)**	**Reference**	**Fragment sizes**	**Reference**
Bcl I	Intron 18	p114.12	0.8/1.1	193	142/99+43	202
Bgl I	Intron 25	Probe C	5.0/20.0	194		
Xba I	Intron 22	p482.6	4.8(1.4)/6.2	195	68+28/96	202
Hind III	Intron 19	F8	2.6/2.7	196	469+167+81/469+248	203
Msp I (a)	3' flanking	p625.3	4.3+3.2/7.5	197		
Msp I (b)	Intron 22	Probe B	3.8/4.0	198		
Bgl II	DXS15	DX13	2.8/5.8	190		
Taq I/Msp I	DXS52	ST14	Multiple	191	60bp repeat	204
Msp I	DXS115	767	6.0+5.8/11.8	199		
Acc I	DXS115	767	4.0/9.0	199		
Pst I	DXS115	767	1.75/1.8	200		
BstX I	DXS115	767	4.25/6.4	150		
Taq I	5' flanking	701.1	4.0/9.5	201		
Intron 7	-	-	-	-	Single bp alteration 'G' or 'A'	205
Intron 13	-	-	-	-	$(CA)_n$ Repeat	206

Table 9. Factor VIII RFLP's - racial variation.

White RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Bcl I	133	0.8/1.1	0.71/0.29	42	193
Bgl I	52	5.0/20	0.90/0.10	18	194
Xba I	88	4.8/6.2	0.59/0.41	48	195
Hind III	56	2.6/2.7	0.30/0.70	42	196
Msp I (a)	67	4.3+3.2/7.5	0.32/0.68	43	197
Msp I (b)	42	3.8/4.0	1.0/0	0	198
Bgl II	NR	2.8/5.8	0.5/0.5	50	190
Taq I/Msp I	NR	Multiple	-	80	191
Msp I	74	6.0+5.8/11.8	0.14/0.86	24	199
Acc I		4.0/9.0	0.90/0.10	18	199
Pst I		1.75/1.80	0.23/0.77	35	200
BstX I	51	4.25/6.4	0.14/0.86	24	152
Taq I	NR	4.0/9.5	0.28/0.72	40	201
Intron 7	225	'G' or 'A'	0.79/0.21	33	205
Intron 13	159	Variable - VNTR	-	91 (Observed)	206

American Black RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Bcl I	28	0.8/1.1	0.17/0.83	28	194
Bgl I	50	5.0/20	0.74/0.26	38	194
Hind III	91	2.6/2.7	0.78/0.22	34	203
Msp I (b)	30	3.8/4.0	1.0/0	0	198

Asian/Indian RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Bcl I	33	0.8/1.1	0.66/0.34	45	194
Bgl I	36	5.0/20	0.94/0.06	8	194
Hind III	80	2.6/2.7	0.29/0.71	41	203
Msp I (b)	28	3.8/4.0	0.93/0.07	13	198

Chinese RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Bcl I	133	0.8/1.1	0.82/0.18	30	207
Xba I	49	4.8/6.2	0.57/0.43	49	207
Bgl I	54	5.0/20	1.0/0	0	207
Hind III	67	2.6/2.7	0.24/0.76	36	203

Table 9. continued.

Japanese RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Bcl I	144	0.8/1.1	0.70/0.30	42	208
Xba I	130	4.8/6.2	0.59/0.41	48	208
Bgl I	138	5.0/20	0.91/0.09	16	208
Msp I (b)	49	3.8/4.0	0.65/0.35	45	198
Bgl II	108	2.8/5.8	0.84/0.16	27	208
Taq I/Msp I	112	Multiple	-	76 (Observed)	208

Malay RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Bcl I	68	0.8/1.1	0.79/0.21	33	203
Hind III	64	2.6/2.7	0.24/0.76	36	203

Polynesian RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Bcl I	121	0.8/1.1	0.55/0.45	49	209
Xba I	115	4.8/6.2	0.5/0.5	50	209
Bgl I	120	5.0/20	0.01/0.99	2	209

Maori RFLP	No. X chromosomes analysed	Alleles (kb)	Frequency	Heterozygosity (%)	Reference
Bcl I	72	0.8/1.1	0.66/0.44	44	209
Xba I	72	4.8/6.2	0.57/0.63	49	209
Bgl I	72	5.0/20	0.06/0.94	11	209

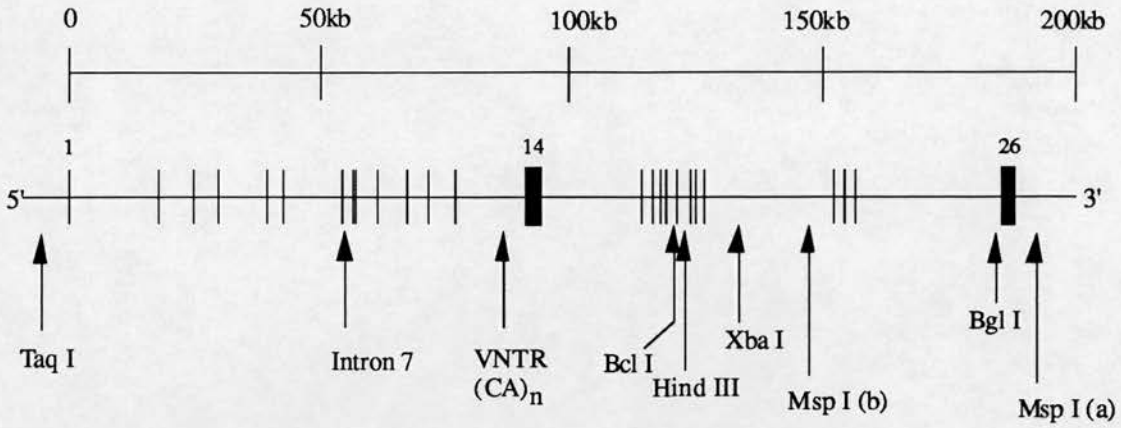


Figure 9. Map of the FVIII gene showing the approximate positions of the intragenic and flanking RFLP's.

1.7.6.1. Intragenic RFLP's.

A number of intragenic polymorphisms have been reported within the FVIII gene although they show linkage disequilibrium and racial segregation and their use is, therefore, dependent upon the allelic frequencies within the population studied.

1. Bcl I: situated within intron 18 and comprising a two allele system. The polymorphism is an excellent marker as over 40% of Caucasian women are predicted to be heterozygotes (Table 9).

2. Bgl I: situated within intron 25, 2kb 5' of exon 26 it detects a two allele system. It is useful for carrier detection studies particularly in the American Black population. In Europeans, a degree of linkage disequilibrium exists between the Bgl I and Bcl I sites with the result that the combined use of both polymorphisms does not significantly increase the yield of informative families.

3. Xba I: situated within intron 22, approximately 18kb 3' to the Bcl I site it again detects

a two allele system. A degree of linkage disequilibrium exists between this site and the Bcl I polymorphism but approximately 25% of females homozygous at the Bcl I site will be informative. Unfortunately the 9.6kb FVIII genomic probe (p482.6) cross-hybridises to a non-FVIII region. Therefore, a double digestion with Kpn I and Xba I is required to move the polymorphic FVIII band away from the non-FVIII band.

4. Hind III: situated within intron 19 it detects a two allele system. The polymorphism is found in approximately 30% of Caucasians but a high degree of linkage disequilibrium exists between this site and the Bcl I site making it of limited use.

5. Intron 7: situated in intron 7 of the FVIII gene, this polymorphism ('A' or 'G') is detected only by PCR. The allelic frequencies are:'G' - 79%; 'A' - 21%). This polymorphism shows strong linkage disequilibrium with both the Bcl I and Xba I polymorphisms and, therefore, yields little additional information.

6. Msp I (b): situated in intron 22 - it detects a two allele system with bands of 4.0 and 3.8kb. This polymorphism is useful only in Japanese families as Caucasians are homozygous.

7. Intron 13 VNTR: a recently reported tandem repeat $(CA)_n$ situated in intron 13 has been shown to be highly informative with 91% of females being heterozygotes. This VNTR is analysed by PCR amplification followed by electrophoresis in polyacrylamide gels. Analysis reveals 8 allelic bands with the number of repeats varying from 16-24.
Excluding the intron 13 VNTR, approximately 70% of females will be heterozygous for one or more polymorphisms with an extremely small risk (<0.1%) of recombination and therefore, of misdiagnosis. If the VNTR is analysed exclusively, then over 90% of women will be informative and it represents the most useful polymorphic marker currently available.

1.7.6.2. Extragenic (Linked) RFLP's.

A number of linked polymorphic markers have proved useful in carrier detection studies in haemophilia A:

1. Bgl II: this was the first of the polymorphic markers to be used in carrier detection and prenatal diagnosis in haemophilia A. Although tightly linked to the FVIII gene, there is a risk of recombination of 5%.

2. Taq I/Msp I: Taq I digestion of genomic DNA and probing with the ST14 probe reveals two distinct polymorphic systems. The primary system is composed of at least 24 alleles ranging in size from 15kb to 3.0kb. The secondary system involves the presence of a Taq I site in a 5.5kb band which gives rise to two bands of 4.0kb and 1.5kb although in the Caucasian population this extra Taq I site is rare. In an unrelated black population, 84% heterozygosity was observed. In addition the ST14 probe is known to recognise a two allele Msp I RFLP in the Caucasian population.

3. Msp I (a): situated 3' to exon 26, and 9kb 3' to the Bgl I RFLP, it detects a two allele system with DNA fragments of 7.5 and 4.3/3.2kb. This Msp I polymorphism is in linkage disequilibrium with all previously described polymorphic sites suggesting it may be of limited value.

3. Taq I: situated 17kb 5' to exon 1, it detects a two allele system with bands of 4.0 and 9kb. However, it shows strong linkage disequilibrium with the Bcl I polymorphism.

Summary.

As with the FIX RFLP's, racial differences exist in the prevalence of the various FVIII polymorphic sites (Table 9). The Bgl I polymorphism is unhelpful in the Chinese but has been shown to be particularly useful in the American Black population. Similarly, the Msp I(b) RFLP is unhelpful in Caucasians and American Blacks but extremely useful in the Japanese population. These differences emphasise the importance of using RFLP's appropriate to the population under study.

The intron 13 VNTR should prove to be extremely useful in carrier detection studies in haemophilia A and in particular may eliminate the need to analyse extragenic markers in some families. No information is available on the combined use of the VNTR with the other intragenic RFLP's although it is probable that they will show some linkage disequilibrium. However, in non-informative females the linked Bgl II and Taq I RFLP's may still prove useful.

1.7.7. Kindred Specific Defects.

Defective synthesis of FVIII may result from a number of gene defects, a number of which are shown in Tables 10 and 11. Early studies used biased techniques eg. screening with Taq I which contains within its recognition sequence the CpG sequence and which, therefore, detected repetitive mutations at a few CpG sites. Tuddenham and colleagues have recently published a comprehensive review of mutations in haemophilia A and report an enormous diversity of mutations ranging from single base-pair substitutions to major deletions.[210]

Although over 150 different mutations have now been reported in Haemophilia A, in a recent paper approximately 50% of the mutations in severe haemophilia A did not occur within the promoter region, the splice junctions, the coding sequence or the polyadenylation site.[211] It is probable that the mutations in these individuals occur

outside the regions studied and the authors speculate that these may include sequences deep within introns, other sequences outside the gene that are important for its expression or another gene involved in factor VIII expression that is closely linked to the factor VIII gene. The molecular basis of haemophilia A is likely, therefore, to be more complex than solely mutations within the FVIII gene.

1.7.8. Mutations in the FVIII gene and Inhibitor Formation.

In the review by Tuddenham et al, inhibitors were found in 12 patients with point mutations.[210] Ten of these had nonsense mutations and were associated with severe disease whilst the remaining two point mutations (2209 Arg-Gln and 2229 Trp-Cys) resulted in a mild-moderate phenotype. Although there appears to be some association between specific mutations and the presence of inhibitors eg. 8 of the 12 inhibitor patients had nonsense mutations at either 1941 or 2147, it is clear that possession of a mutation by itself is insufficient to specify inhibitor formation and the reasons for this are unclear. However, it is apparent that deletions within the FVIII gene appear to be associated with an approximate five-fold higher risk of developing inhibitors compared to other severe haemophiliacs without gene deletions.

Table 10. Deletions/Insertions and the FVIII gene.

Mutation	Exon	VIII:C (iu/dl)	Inhibitors
39kb[212]	23-25	<0.01	+
60kb[213]	11-19	<0.01	+
7kb[214]	6	<0.01	-
2.5kb[214]	14	<0.01	-
5.5kb[1214]	22	<0.05	-
16kb[214]	23-25	<0.01	-
7kb[214]	24-25	<0.01	-
>35kb[216]	1-5	<0.01	-
2.0kb[217]	3	<0.01	-
15-20kb[217]	7-10	<0.01	+
2.0kb[217]	14	<0.01	-
12-14kb[217]	14	<0.01	+
NR[217]	26	<0.01	-
NR[218]	23-26	<0.01	+
>210kb[219]	1-26	<0.01	-
15kb[220]	15-18	<0.01	+
>127kb[221]	1-22	<0.01	+
>2kb[222]	26	<0.01	-
22kb[223]	26	<0.01	-
Insertion of L1 Sequence			
3.8kb[224]	14	NR	NR
2.3kb[224]	14	NR	NR

NR - not recorded.

Table 11. Point mutations within the FVIII gene.

Mutation/Codon	Intron/Exon	VIII:C (iu/dl)	Inhibitors
TCGA-TCAA[225]	4/-	0.05-0.10	-
TCGA-TTGA[212]	2/-	<0.01	-
TCAA-TCGA[212]	25/-	<0.01	-
CGAA-CGGA Glu-Gly272[226]	/7	0.12	-
CGA-TGA Arg-Stop 336[227]	-/8	<0.01	-
CGC-TGC Arg-Cys 372[228]	-/8	0.04	-
CGC-TGC Arg-Cys 1689[227]	-/14	<0.01	-
CGA-TGA Arg-Stop 1960[229]	-/18	<0.01	+
CGA-TGA Arg-Stop 1960[194]	-/18	<0.01	-
CGA-TGA Arg-Stop 2147[230]	-/23	<0.01	NR
CGA-CCA Arg-Pro 2116[230]	-/22	<0.01	NR
CGA-TGA Arg-Stop 2147[231]	-/23	<0.01	+
CGA-TGA Arg-Stop 2209[212]	-/24	<0.01	-
CGA-TGA Arg-Stop 2209[211]	-/24	<0.01	-
CGA-TGA Arg-Stop 2209[197]	-/24	<0.01	-
CGA-TGA Arg-Stop 2209[231]	-/24	<0.01	+
CGA-CAA Arg-Gln 2209[231] -	/24	<0.01	-
CGA-CAA Arg-Gln 2209[232]	-/24	<0.01	-
CGA-CAA Arg-Gln 2209[233]	-/24	<0.01	-
CGA-TGA Arg-Stop 2307[212]	-/26	<0.01	+
CGA-TGA Arg-Stop 2307[234]	-/26	<0.01	-
CGA-TGA Arg-Glu 2307[234]	-/26	0.09	-

NR - not recorded; Arg - arginine; Gln - glutamine; Pro - proline; Cys - cysteine; Glu - glutamic acid; Gly - glycine; Stop - stop codon.

1.8. Recent Advances in Molecular Biology.

In the last 4-5 years, a number of techniques have been developed which have considerably simplified both the molecular characterisation of FVIII and FIX mutations and the detection of carriers by either RFLP analysis or direct detection of the underlying mutations.

Such techniques can be grouped into: (1) methods for detecting large deletions or major rearrangements within a gene eg. multiplex amplification;[235] (2) methods which define the approximate site of mutation eg. Ribonuclease A (RNAse) cleavage,[236] denaturing gradient gel electrophoresis (DGGE),[237] chemical mismatch analysis;[238] and (3) sequencing methods that delineate the exact base alterations responsible for the disease eg. direct sequencing of amplified DNA.[239] Many of the these techniques use the Polymerase Chain Reaction (PCR) technique[240] to selectively amplify small regions of the gene.

1.8.1. Polymerase Chain Reaction (PCR).

Southern blotting although superior to previously described molecular hybridisation techniques relies upon many steps: restriction enzyme digestion, agarose gel electrophoresis, blotting, preparation of probes etc.. Such a technique, whilst producing excellent results, is time consuming, labour intensive and relatively costly in terms of reagents. In late 1985, Saiki et al, reported a technique by which specific DNA fragments are enzymatically amplified *in vitro* by a DNA polymerase using short synthetic oligonucleotide primers flanking the area of interest to direct the enzyme to the appropriate region. Repeated cycles of amplification result in an exponential increase in DNA. The use of a heat-stable DNA polymerase (*Thermus aquaticus*) not only reduces background amplification due to non-specific binding of oligonucleotide primers but permits longer segments of DNA to be amplified. The technique allows the use of small amounts of DNA eg. chorionic villi without purification and is readily automated. Amplified products may be analysed in a number of ways including electrophoresis in

agarose and polyacrylamide gels, restriction mapping and direct sequencing.

1.8.2.1. The Use of PCR in Haemophilia A.

In 1987, Kogan et al[202] described the use of the PCR technique to amplify the regions of the FVIII gene containing the Bcl I and Xba I polymorphisms and subsequently to directly analyse a polymorphism in intron 7.[205] Following amplification the DNA is digested with the appropriate enzyme, electrophoresed, stained with ethidium bromide and visualised with UV light. Depending upon the presence or absence of the appropriate restriction site and the sex of the individual either one or more bands are observed.

Although the technique is valuable in carrier detection and the prenatal diagnosis of haemophilia A, its use is limited for the same reasons as Southern blotting, namely that 30% of women will be homozygous for either the Bcl I or Xba I RFLP's. In addition, the Xba I amplifications generate a non-FVIII band which masks one of the polymorphic bands making its use with PCR limited. However, alternative strategies for carrier detection using PCR may be possible eg. detecting polymorphisms that do not alter restriction sites.[241] A number of the FVIII polymorphisms can now be analysed by PCR (see Table 8).

In addition to studying FVIII gene polymorphisms, Kogan and colleagues were also able to sex individuals using Y-specific primers allowing the rapid sexing of fetuses in 'at-risk' pregnancies.[202]

Subsequent developments of the PCR technique have allowed the identification of a number of mutations within the FVIII gene. In 1988, Gitschier and colleagues used the PCR technique to screen haemophiliacs for mutations at FVIII cleavage sites.[227] Codons encoding four of the six arginine residues present at these sites contained CpG

dinucleotides and were, therefore, considered potential hotspots for mutation. This approach led to the discovery of mutations at both the activated Protein C and thrombin cleavage sites in haemophilia A patients.

1.8.2.2. The Use of PCR in Haemophilia B.

The PCR technique can be used in the detection of haemophilia B carriers in one of several ways:

1. The amplification of specific polymorphisms (see Table 5).

2. Direct detection of mutations. The relatively small size of the FIX gene allows the design of primers to selectively amplify all of the FIX gene, generating sufficient material for direct sequence analysis. This is superior to the use of RFLP's but requires considerably more time and effort. However, the use of this technique should allow accurate detection of carriers and rapid antenatal diagnosis in almost all 'at-risk' females.

3. Chemical mismatch analysis. This technique permits rapid screening for mutations by detecting the formation of heteroduplexes. Briefly, radio-labelled amplified wild-type DNA and amplified mutant DNA are mixed, denatured and allowed to reanneal. If a mutation is present, a mismatch occurs which after chemical modification is chemically cleaved and the products of the cleavage detected by electrophoresis. The extra bands generated by the cleavage indicating the approximate position of the mutation which can then be confirmed by direct sequencing. Such a technique is readily applicable to the study of mutations in the FIX genes of haemophilia B families.[242]

1.9. Carrier Detection in the Haemophilias.

Haemophilia A and B are both X-linked recessive diseases and as a result genetic counselling should be offered to 'at-risk' females. Carrier detection is generally undertaken for 2 reasons:

1. An affected individual exists within a family and other female members wish to know whether they may be carriers and, therefore, at risk of producing a similarly affected child.

2. A female with no family history of haemophilia gives birth to an affected child and wishes to know whether there is a risk to subsequent children.

Carrier detection is approached by:

A. Pedigree analysis

B. Phenotypic analysis

C. Genotypic analysis (reviewed in Chapter 4: Discussion)

and by combining these results it may be possible to establish the probability of carriership in a specific female.

1.9.1. Pedigree Analysis.

An accurate family pedigree forms an essential part of the initial investigation of any family with a bleeding diathesis. As well as information about possible linkage of the haemophilic gene, details about other X-linked traits eg. G6PD deficiency, colour blindness should be sought.

On the basis of this pedigree data a female may be classified as either an obligate carrier, a potential carrier or normal.

Obligate carriers include:

a. The daughter of a haemophiliac.

b. A mother with two affected children.

c. A mother with one affected child plus a family history of haemophilia.

d. The mother of two daughters, each of whom has an affected male (assuming their father is normal).

Possible carriers include:

a. A female who has no affected children but a male haemophilic relative on the maternal side.

b. The mother of a single haemophilic child.

An accurate pedigree, therefore, forms an essential part of genetic counselling and may in some families, allow a female to be excluded as a carrier.

1.9.2. Phenotypic Analysis.

Traditionally, the laboratory detection of carriers of haemophilia A and B has depended upon the results of coagulation testing. The earliest attempts at carrier detection involved the measurement of FVIII and FIX clotting activity. In normal individuals the levels of VIII:C and IX:C are generally within the range 0.5-2iu/dl with a mean value of 1.0iu/dl. In male haemophiliacs the levels are reduced in proportion to the severity of the disease. Consistent with this, levels of VIII:C and IX:C will tend to be lower in carriers with average levels of 0.5iu/dl. However, the actual levels can vary widely depending upon the degree of X-chromosome inactivation which has occurred - Lyon's hypothesis.[243] Lyon's hypothesis of random X-chromosome inactivation postulates that at an early stage in embryological development one of the X-chromosomes becomes inactivated, a process which is random and is irrevocable for the descendants of each cell after the decision has been made. Lyonisation, therefore, results in some carriers with a markedly reduced level of VIII:C and others with a value in the normal range (Figure 10). It is this latter group in whom carrier detection, based upon phenotypic data, is extremely difficult. Recently, a report has appeared in which X-chromosome inactivation has occurred in affected

females but which does not appear to be random ie. preferential inactivation of the normal X-chromosome.[244] It is possible that this apparent non-random X-inactivation represents an ascertainment bias. Such biases arise when the study sample is ascertained through affected individuals (as usually happens in studies of diseases) rather than by analysing a random sample of the population. In this case, therefore, the suggestion that there is preferential inactivation of the normal X-chromosome may be a chance finding rather than a true abnormality.

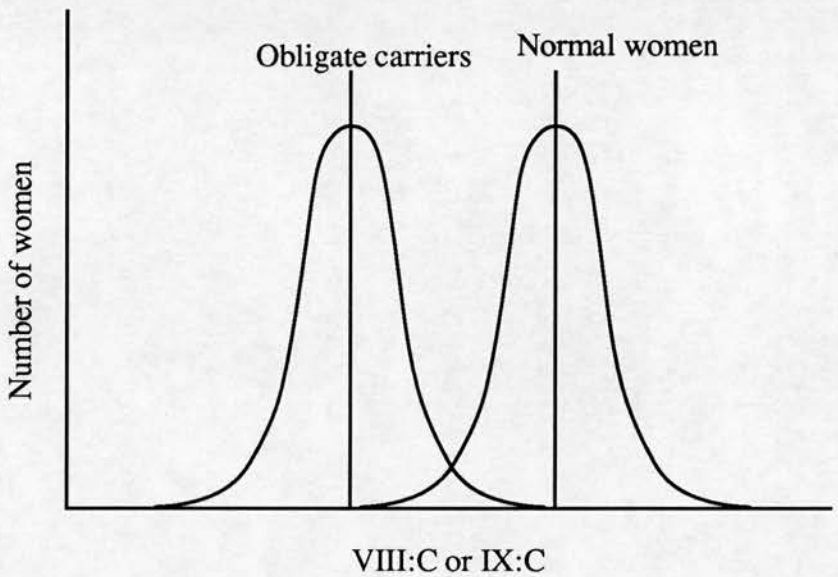


Figure 10. VIII:C (or IX:C) values in normal women and obligatory carriers of haemophilia demonstrating the overlap that occurs between normal women with low VIII:C (or IX:C) and obligatory carriers with normal VIII:C (or IX:C).

In addition to Lyonisation a number of other factors have been shown to affect the measurement of VIII:C, IX:C and vWF in plasma and must be considered when interpreting the results of coagulation testing:[245]

1. Age - VIII:C levels increase with age with the highest values being found in the very young and very old and the lowest values are found in the 25-30 year-old age range.

An International Co-operative study in 1986[246] concluded that age had a significant effect on both VIII:C and vWF:Ag with the values being highest at the extremes of age.

2. ABO blood group - females with blood groups A, B and AB, both normal and carriers have significantly higher levels of both FVIII and vWF although the primary effect is upon vWF.[246] It is unclear how the genes coding for the ABO blood group system (located on chromosome 9) can affect the expression of a different chromosome although it is possible that the effect may occur post-translationally.

3. Oestrogen therapy/pregnancy are well recognised to increase both VIII:C and vWF.[247]

4. Laboratory variation - a number of variables have been shown to affect the assay of VIII:C, IX:C and vWF and these include:

A. The collection of the sample and its subsequent processing. VIII:C is relatively labile and samples must, therefore, be assayed immediately. VIII:Ag appears to be more stable, samples can be frozen and processed in the future. Samples for IX:C and vWF assays can also both be safely frozen.

B. Method of assay ie. one-stage or two-stage assay for VIII:C.

C. The choice of reagents/standards.

D. Analyst.

It is important, therefore, for individual laboratories to establish both normal ranges for their own local population and to standardise their assays using the various national and international standards available.

1.9.2.1. Historical Review of Carrier Detection in Haemophilia A.

The first attempt at carrier detection in the female relatives of haemophiliacs was reported by Schloessmann in 1930[248] who examined 34 carriers of haemophilia of whom 16 had an abnormal bleeding tendency. In 13 he measured the coagulation time and found it to be abnormal in 11. Andreassen in 1943[249] also found a tendency to haemorrhage in female carriers and demonstrated abnormal coagulation in 30 out of 31 cases. Sköld, however, in 1944[250] measured the whole blood clotting time and was unable to demonstrate any difference between carriers and normal females. In 1951, Merskey et al[251] using the whole blood clotting time, prothrombin consumption test and an AHF titration test (to measure VIII:C) failed to demonstrate any abnormality between obligate carriers and a control group of normal females. Two years later the first accurate quantitative measurement of VIII:C was published by Graham,[252] who found low levels in six out of ten carriers of mild haemophilia in a single large family and attributed this to a peculiarity of the haemophilic gene responsible for the disease. In apparent confirmation, Gardikas et al[253] reported normal levels of VIII:C in ten obligatory carriers of severe haemophilia whilst Margolius and Ratnoff in 1956[254] detected an abnormal value in only one of nineteen carriers.

Numerous subsequent studies by many groups have demonstrated that diminished levels of VIII:C may be found in some carriers of both mild and severe haemophilia but that there is a wide variation in the results obtained with some obligate carriers having entirely normal activity and some normal women having borderline low VIII:C values. It is now accepted that the measurement of VIII:C alone provides a poor means of carrier detection.

The results of the published studies of VIII:C levels in carriers of haemophilia A are shown in Table 12. Of interest are the widely differing VIII:C values obtained by many workers; the differences in activity values between races; the discrepancies between 1-stage and 2-stage assays and whether the samples were assayed immediately or after

freeze/thawing. These results emphasise the importance of standardising laboratory techniques to minimise such errors.

Following the introduction of specific assays for VIII:C the next significant advance in the detection of haemophilia A carriers was the report in 1971[255] of a specific assay for FVIII related antigen (VIIIR:Ag=vWF:Ag) or protein. Zimmerman found the ratio between VIII:C and vWF:Ag (VIIIR:Ag) in female carriers of haemophilia A was significantly lower than in normal females and that this ratio (VIII:C/vWF:Ag) provided a better criterion for detecting carriers than VIII:C alone. Zimmerman's results were particularly interesting as they showed that carriers with an VIII:C of more than 100% had a proportional increase in their vWF:Ag (VIIIR:Ag) level. Nevertheless, the ratio of activity to antigen was still considerably less than in normal women and on the basis of this carriers could still be distinguished. Although subsequent work has confirmed the validity of these measurements in the clarification of carrier status, there continues to exist a wide variation in the observed differences in carrier detection between various laboratories.

A co-operative study in 1977 suggested that variation in the laboratory estimation of VIII:C and vWF:Ag was related to laboratory technique rather than statistical methods.[256] Seligshon in 1979[257] suggested that in cases where carrier status was in doubt the repeated measurement of VIII:C and vWF:Ag was necessary to eliminate errors inherent in the assays. Graham in 1980[258] showed that discriminants useful for detecting carriers of severe haemophilia A were associated with large error rates when applied to carriers of mild haemophilia A because of the greater overlap between the factor-VIII related activities of mild and normal women. In 1977 a WHO Memorandum on carrier detection in haemophilia[245] suggested that the measurement of vWF:RCo might replace vWF:Ag whilst Marshall and Stenhouse in 1979[259] advocated that all three tests be used. Shen in 1982[260] compared the predictive ability of 28 different discriminant equations which he

calculated from various ratios of the three tests. He found that vWF:RCo was not as good as vWF:Ag for carrier detection and these findings were confirmed by Duncan in 1984.[261]

The results of carrier detection studies using the measurement of VIII:C, vWF:Ag and vWF:RCo together with the various methods of statistical analysis are reviewed and summarised in Table 13.

1.9.2.2. The Value of VIII:Ag Assays in the Detection of Haemophilia A Carriers.

In 1978, Peake et al[109] described an immunoassay for VIII:Ag and reported its subsequent use in carrier detection. They found that a comparison of VIII:Ag/vWF:Ag (VIIIC:Ag/VIIIR:Ag) ratios and VIII:C/vWF:Ag (VIII:C/VIIIR:Ag) ratios gave the same proportional misclassification of carriers as normal (4/23) but that the former ratio showed greater discriminatory power. There was no statistical difference between the two ratios obtained in carriers of mild or severe haemophilia. An advantage of the VIII:Ag measurement is its relative stability in plasma allowing samples to be batched and processed at a later date. Hoyer in 1982[262] used the measurement of VIII:Ag and vWF:Ag in the detection of pregnant haemophilia carriers and was able to demonstrate that it was as useful in discriminating between carriers and non-carriers as the measurement of the VIII:C/vWF:Ag ratio.

1.9.2.3. Summary of Phenotypic Methods for the Detection of Carriers of Haemophilia A.

It is generally accepted than the measurement of VIII:C alone does not provide sufficient discrimination between normal and obligatory carriers of haemophilia A. Currently phenotypically based carrier detection using the measurement of VIII:C and vWF:Ag allows the classification of 85% of potential haemophilia A carriers. However, it is clear that in some true carriers, Lyonisation will result in normal VIII:C values, a normal VIII:C/vWF:Ag ratio and therefore, in misclassification. It is these individuals in whom molecular based methods of carrier detection are likely to play an important role.

Table 12. VIII:C values in obligatory carriers, potential carriers and normal women.

Author First Author	Year	Assay	VIII:C activity (%) Obligate carriers No.	Range	Mean	Possible carriers No.	Range	Mean	Normal No.	Range	Mean
Merskey[251]	1951	WBCT, PCT & AHG	12	No difference from control		8	No difference from control		21		
Graham[252]	1953	AHG 2-stage	8	60-88	70	-	-	-	10	68-149	96
Margolius[254]	1956	AHG 2-stage	19	1 abnormal found		8	No abnormal results		-	-	-
Douglas[263]	1957	AHG 2-stage & PCT	2	17-24	20	-	-	-	-	-	-
Taylor[264]	1957	AHG 2-stage	79								
Gardikas[253]	1957	AHG 2-stage	10	80-105	87	-	-	-	-	-	-
Didisheim[265]	1958	AHG 2-stage	63	10-120	76	-	-	-	80	45-220	100
		& PCT	14	10-100	62	-	-	-	80	45-150	100
Pitney[266]	1959	AHG 2-stage	16	26-100	-	9	53-160	-	40	60->200	-
Ikkala[267]	1960	AHG 1-stage	4	40-55	55	-	-	-	-	-	-
Bentley[268]	1960	EPCT	26	30-40	-	-	-	-	51	50-200	-
Rapaport[269]	1960	AHG 2-stage	35	22-135	58	-	-	-	30	52-133	92
Githens[270]	1962	AHG + Correction	6/12 abnormal			5/19 abnormal		-	-	-	-
Nilsson[271]	1962	AHG 1-stage	33	15-74	38	45	-	-	39	56-130	90
Goudemand[272]	1962	AHG	17	15-100	50	-	-	-	46	47-133	86
Bradlow[273]	1962	AHG	6			4	-	-	-	-	-
Deutsch[274]	1962	AHG 1-stage	10	14-55	47	-	-	-	109	50-205	103
Mulder[275]	1964	AHG 1-stage	17	24-60	48	-	-	-	17	-	100
Miller[276]	1963	AHG 1-stage	17	25-125	51	15	-	-	30	80-210	112
Kerr[277]	1966	AHG 2-stage	29	26-126	50	43	26-150	71	99	32-200	95
Gugler[278]	1965	AHG 1-stage	28	16-110	45	-	-	-	30	65-220	112
Bergna[279]	1964	AHG 1-stage	12	36-100	69	-	-	-	15	61-147	94

WBCT - Whole blood clotting time; AHG - antihaemophilic globulin; EPCT - erythrocyte consumption time; PCT - prothrombin consumption test.

Table 12 continued. VIII:C values in obligatory carriers, potential carriers and normal women.

Author First Author	Year	Assay	VIII:C activity (%) Obligate carriers No.	Range	Mean	Possible carriers No.	Range	Mean	Normal No.	Range	Mean
Bennett[280]	1970	AHG 1-stage	6	36-52	46	3	23-90	66	5	50-100	50
Zimmerman[255]	1971	AHG 1-stage	25	18-180	67	-	-	-	22	60-170	-
Ekert[281]	1973	AHG 1-stage	13	16-136	78	21	22-195	84	20	60-215	116
Bennett[282]	1973	AHG 1-stage	-	-	-	18	25-145	-	38	55-210	-
Denson[283]	1973	AHG 2-stage	18	25-75	-	-	-	-	18	50-120	-
Bouma[284]	1975	AHF 1-stage	22	-	-	19	33-159	75	30	-	-
Meyer[285]	1975	VIII:C 1-stage	49	-	61	-	-	-	31	-	94
Eyster[286]	1976	VIII:C 1-stage	7	-	58	-	-	-	20	-	108
			10	-	52	7	-	94	7	-	105
Rizza[287]	1975	VIII:C 2-stage	24	22-116	55	-	-	-	34	44-63	96
Prentice[288]	1975	VIII:C 1-stage	25	20-150	-	32	20-168	-	23	50-130	-
Hoyer[289]	1975	VIII:C 1-stage	24	40-70	53	-	-	-	22	60-170	101
			24	25-105	60	-	-	-	32	45-21-	103
Graham[290]	1976	VIII:C 1-stage	4	9-112	66	-	-	-	-	-	-
Hathaway[291]	1976	VIII:C 1-stage	33	-	72	51	-	108	37	-	-
Weinstein[292]	1976	VIII:C 1-stage	19	15-107	48	-	-	-	28	55-175	99
Gomperts[293]	1976	VIII:C 2-stage	11	-	-	-	-	-	15	70-195	118
			10	-	-	-	-	-	15	47-130	94
Klein[294]	1977	VIII:C 1 & 2 stage	35	10-143	62	-	-	-	21	58-224	105
Ratnoff[295]	1977	VIII:C 1-stage	87	-	55	-	-	-	109	-	97
Seligsohn[257]	1979	VIII:C 1-stage	37	25-105	62	-	-	-	48	65-200	111
Graham[258]	1980	VIII:C 1-stage	15	18-270	86	-	-	-	13	81-400	148
Peake[296]	1981	VIII:C 1-stage	23	9-130	57	-	-	-	26	42-150	82

AHF - antihaemophilic factor; AHG - antihaemophilic globulin.

Table 12 continued. VIII:C values in obligatory carriers, potential carriers and normal women.

Author First Author	Year	Assay	VIII:C activity (%) Obligate carriers No.	Range	Mean	Possible carriers No.	Range	Mean	Normal No.	Range	Mean
Mibashan[297]	1981	VIII:C 1-stage	21	-	60	-	-	-	28	-	131
Shen[260]	1982	VIII:C 1-stage	37	17-110	47	-	-	-	31	55-170	95
Wahlberg[298]	1982	VIII:C 1 & 2	18	24-120	63	43	15-124	69	40	53-118	81
		stage	18	32-109	59	43	23-104	73	40	46-133	74
Duncan[261]	1984	VIII:C 1 & 2	50	20-140	-	-	-	-	50	55-12	-
Green[299]	1986	VIII:C various	336						37	-	-
Graham[300]	1986	VIII:C various	296	52-72	54	-	-	-	137	84-117	-
Hellstern[301]	1987	VIII:C 1-stage	5	45-65	54	-	-	-	102	65-210	-
Percy[302]	1988	VIII:C 1-stage	42	-	-	28	-	-	40	-	-

Table 13. Phenotypic analysis in obligate carriers of haemophilia A.

Author First author	Year	VIII:C Assay	vWF technique	Statistical method	Obligate carriers Number	Correctly classified Number	%	Misclassified Number
Zimmerman[255]	1971	AHG 1-stage	EIA	Ratio	25	23	92	2
Ekert[281]	1973	AHG 1-stage	Clotting	Ratio	13	10	77	3
Bennett[282]	1973	AHG 1-stage	Clotting	Ratio	6	6	100	-
Denson[283]	1973	AHG 2-stage	IEP	Ratio	18	15	83	3
Ridgway[303]	1973	NR	IEP	Lin Regr Anal	9	9	100	-
Bouma[284]	1975	AHG 1-stage	IEP	Log Disc Anal	22	18	82	4
Meyer[285]	1975	VIII:C 1-stage	IEP	Log Disc Anal	49	40	82	9
Eyster[286]	1976	VIII:C 1-stage	IEP	Lin Disc Anal	17	16	94	1
Holmberg[304]	1975	VIII:C 1-stage	IEP	Ratio	23	16	70	7
Rizza[287]	1975	VIII:C 2-stage	IEP	Ratio	34	25	73	9
Prentice[288]	1975	VIII:C 1-stage	IEP	Lin Regr Anal	26	23	88	3
Hoyer**[289]	1975	VIII:C 1-stage	RIA	Lin Regr Anal	8	7	88	1
Panicucci[305]	1975	NR	IEP	Ratio	56	55	98	1
Graham[290]	1976	VIII:C 1-stage			4			
Hathaway[291]	1976	VIII:C 1-stage	IEP	Ratio	33	29	88	4

** Carrier detection in Pregnancy.

Antigen techniques: EIA - electroimmunoassay; IEP - immunoelectrophoresis; RIA - radioimmunoassay; SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis; FIA - fluorimmunoassay; clotting - antigen estimated by capacity of subject's plasma to block clot-inhibitory properties of heterologous antisera against AHG.

Statistical methods: Lin Disc Anal - linear discriminant analysis; Log Disc Anal - logarithmic discriminant analysis; Lin Reg Anal - linear regression analysis; Semi-Log Regr - linear regression of the ratio of log VIII:C/log vWF:Ag versus VIII:C; ratio - ratio of VIII:C to vWF:Ag.

Table 13 continued. Phenotypic analysis in obligate carriers of haemophilia A.

Author First author	Year	VIII:C Assay	vWF technique	Statistical method	Obligate carriers Number	Correctly classified Number	%	Misclassified Number
Weinstein[292]	1976	VIII:C 1-stage	SDS-PAGE	Semi-Log Regr	19	17	89	2
Lusher[306]	1976	NR	IEP	NR	25	25	100	0
Lian[307]	1976	NR	Ristocetin	Ratio	20	18	90	2
Gomperts[293]	1976	VIII:C 2-stage	IEP	Lin Disc Anal	21	20	95	1
Klein[294]	1977	VIII:C 1-stage	EID	Lin Disc Anal	35	26	74	9
(Multicentre)		VIII:C 1-stage	RIA	Lin Regr Anal	35	30	86	5
		VIII:C 1-stage	EIA	Ratio	35	30	86	5
Ratnoff[295]	1977	VIII:C 1-stage	Clotting	Log Disc Anal	87	82	94	5
Seligsohn[257]	1979	VIII:C 1-stage	EIA	Lin Disc Anal	37	30	83	7
Peake[296]	1981	VIII:Ag	EIA	Ratio	23	19	83	4
		VIII:C 1-stage		Ratio	23	19	83	4
Shen[260]	1982	VIII:C 1-stage	EIA/ Ristocetin	Lin Disc Anal	37	35	95	2
Wahlberg[298]	1982	VIII:C 1 & 2 stage	RIA/EIA Ristocetin	Mult Var Anal	18	13	72	5
Duncan[261]	1984	VIII:C 1 & 2 stage	EIA/FIA/ Ristocetin	Mult Var Anal	50	47	94	3
				Lin Disc Anal	50	42	84	8
Green[299] (Multicentre)	1986	VIII:C Various	Various	Lin Disc Anal	336	293	87	43
Percy[302]	1988	VIII:C 1-stage	EIA	Various	42	38	91	4

Antigen techniques: EIA - electroimmunoassay; IEP - immunoelectrophoresis; RIA - radioimmunoassay; SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis; FIA - fluorimmunoassay; clotting - antigen estimated by capacity of subject's plasma to block clot-inhibitory properties of heterologous antisera against AHF.
Statistical methods: Lin Disc Anal - linear discriminant analysis; Log Disc Anal - logarithmic discriminant analysis; Lin Reg Anal - linear regression analysis; Semi-Log Regr - linear regression of the ratio of log VIII:C/log vWF:Ag versus VIII:C; ratio - ratio of VIII:C to vWF:Ag.

1.9.3. Haemophilia B.

Haemophilia B is a more heterogeneous disorder than haemophilia A with roughly one-third of patients having CRM^+ disease. A low IX:C in obligatory carriers of haemophilia B has been shown in a number of studies (Table 14) but as with carriers of haemophilia A, the overlap between normal and reduced activity is wide. To overcome these limitations, the measurement of IX:Ag has been suggested as a method of improving the accuracy of phenotypic based carrier detection. A number of techniques have been employed to measure IX:Ag including electroimmunoassay (EIA),[308,309,310] antibody neutralization[310,312] and radioimmunoassay (RIA).[313]

Elödi[313] found that in 8 obligate carriers of haemophilia (CRM^-), there was good correlation between IX:C and IX:Ag but that the additional measurement of IX:Ag did not improve the detection of carriers. Matsuzo[308] reported that the levels of IX:C in obligate carriers of haemophilia B were significantly lower than those of VIII:C in obligate carriers of haemophilia A. In addition, he noted the discrepancy between IX:C and IX:Ag in haemophilia B_m and CRM^+ patients and carriers and pointed out the value of these measurements in the detection of carriers with these disorders. Thompson[309] found the mean values for both IX:C and IX:Ag significantly lower in heterozygotes compared to a control group but felt that IX:Ag was of limited value in improving detection rates but that it might serve as an independent method to confirm the results of clotting activity assays.

Graham in 1979[314] showed that the most efficient and cost-effective method for classifying CRM^- carriers was univariate discrimination based upon assay of IX:Ag alone. For CRM^+ carriers, bivariate linear discrimination utilising both IX:C and IX:Ag assays was preferable. Ørstavik in the same year[315] concluded that the quantitative determination of both factor IX antigen and factor IX activity was of value in establishing the risk of carriership in both CRM^- and CRM^+ haemophilia B families.

Table 14. IX:C and IX:Ag in obligate carriers of haemophilia B and normal female controls.

Author First author	Year	Assay		Obligate carriers Number	Range(%)	Mean(%)	Normal females Number	Range(%)	Mean(%)
Firkin[316]	1958	TGT + correction		5	Reduced	-	-	-	-
Bond[317]	1959	PTT + correction		4	Reduced	-	-	-	-
Bolton[318]	1959	2-stage IX:C		7	15-103	63	37	68-160	102
Barrow[319]	1960	1-stage IX:C		13	29-109	67	61	55-171	93
Nilsson[271]	1962	1-stage IX:C		18	15-110	48	31	56-160	92
Simpson[320]	1962	2-stage IX:C		53	11-130	55	119	32-170	97
Forta-Pessoa[311]	1962	2-stage IX:C		17	10-100	45	100	20-200	92
Didisheim[312]	1962	1-stage IX:C		14	10-100	62	80	45-100	NR
Gugler[321]	1965	1-stage IX:C		11	20-47	31	30	78-160	108
Elödi[313]	1975	1-stage IX:C	CRM^-	8	14-70	32	20	NR	93
		Ab Neut IX:Ag	CRM^-	8	14-78	34	20	NR	89
Matsuzo[308]	1976	1-stage IX:C	CRM^-	15	18-70	33	21	NR	100
		Ab Neut IX:Ag	CRM^-	4	30-63	44	-	-	-
Thompson[309]	1977	1-stage IX:C	CRM^-	15	39-100	62	15	44-137	86
		RIA IX:Ag	CRM^-	15	42-106	73	15	47-138	93
Kasper[310]	1977	EIA IX:Ag		51	12-119	42	-	-	-
Graham[314]	1979	1-stage IX:C	CRM^-	29	NR	NR			
			CRM^+	18	10-140	NR	20	70-130	NR
		EIA IX:Ag	CRM^-	29	NR	NR			
			CRM^+	18	26-138	NR	20	80-135	NR
Ørstavik[315]	1979	1-stage IX:C	CRM^-	18	38-112	63	40	49-130	94
			CRM^+	10	33-112	60	-	-	-
		EIA IX:Ag	CRM^-	18	20-91	53	40	63-136	95
			CRM^+	10	76-191	113	-	-	-

Ab Neut - antibody neutralisation; EIA - electroimmunoassay; PTT - partial thromboplastin time; RIA - radioimmunoassay; TGT - thromboplastin generation test.

1.10. Bayesian Methods in Risk Estimation.

Bayesian statistics is a method of combining probabilities which is widely used in estimating genetic risks and is, therefore, applicable to the study of both haemophilia A and B.[245] The theorem or law was originally formulated by the Reverend Bayes[322] and enables the overall probability of an event, C such as carrier status to be calculated taking into account the family pedigree data and data derived from various tests eg. biochemical assays, RFLP analyses.

Bayes's theorem may be stated as follows:[323]

1. If the prior probability of an event **C** occurring is denoted as **P(C)** and

2. the prior probability of an event **C** not occurring is denoted as **P(NC)** and

3. the conditional probability of an observation **O** occurring if **C** occurs equals **P(O|C)** and

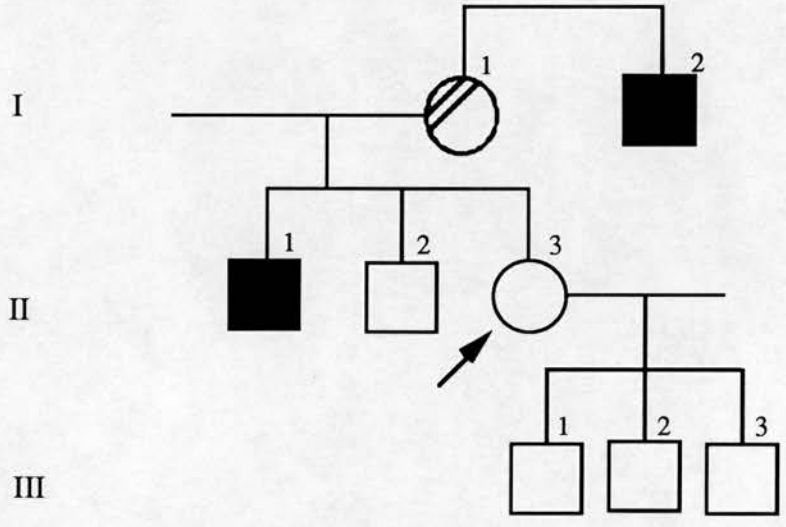
4. the conditional probability of observation **O** occurring if **C** does not occur equals **P(O|NC)**, then the overall probability of event **C** given **O** is observed equals:

$$\frac{P(C) \times P(O|C)}{[P(C) \times PO|C)] + P(NC) \times P(O|NC)]}$$

This is more clearly shown in the Bayesian table on the following page.

Probability	Event C occurs	Event C does not occur
Prior	P(C)	P(NC)
Conditional		
O occurs	P(O|C)	P(O|NC)
Joint	P(C) x P(O|C)	P(NC) x P(O|NC)
Final Probability	$\frac{P(C)\times P(O\|C)}{[P(C)\times P(O\|C)]+[P(NC)\times P(O\|NC)]}$	$\frac{P(NC)\times P(O\|NC)}{[P(C)\times P(O\|C)]+[P(NC)\times P(O\|NC)]}$

The application of this approach to the prediction of carrier status in X-linked recessive diseases such as haemophilia is illustrated below.



In this Pedigree, II-3 is the daughter of an obligate carrier of Haemophilia A and wishes to know whether she is a carrier or not. The prior probability that she is a carrier (from the pedigree data) is 1/2 and the prior probability that she is not a carrier is also 1/2. II-3 has three normal sons - if she is a carrier the conditional probability that all three would be normal is 1/2 x 1/2 x 1/2 = 1/8; if she is not a carrier, the conditional probability that

all three sons would be normal is very nearly 1. It not exactly 1 as there is a risk that a son could be affected because of a new mutation. If she is not a carrier, the conditional probability that all three sons would be normal is, therefore, 1-μ where μ is the mutation rate in females. (For clarity in these examples μ is assumed to extremely small and therefore, ignored).

If we consider the joint probability ie. the product of the prior and conditional probabilities; then the joint probability that she is a carrier is 1/2 x 1/8 = 1/16 and the joint probability that she is not a carrier is 1/2 x 1 = 1/2. The final risk or posterior probability that she is a carrier is, therefore:

$$\frac{1/16}{1/16+1/2}=\frac{1}{9}$$

and the final risk or posterior probability that she is a not a carrier is:

$$\frac{1/2}{1/16+1/2}=\frac{8}{9}$$

Tabulating the above information:

Probability	**II-3 carrier**	**II-3 not a carrier**
Prior Probability	1/2	1/2
Conditional Probability		
3 normal children	1/8	1
Joint Probability	1/2 x 1/8 = 1/16	1/2 x 1 = 1/2
Final Risk or Posterior probability	1/9	8/9

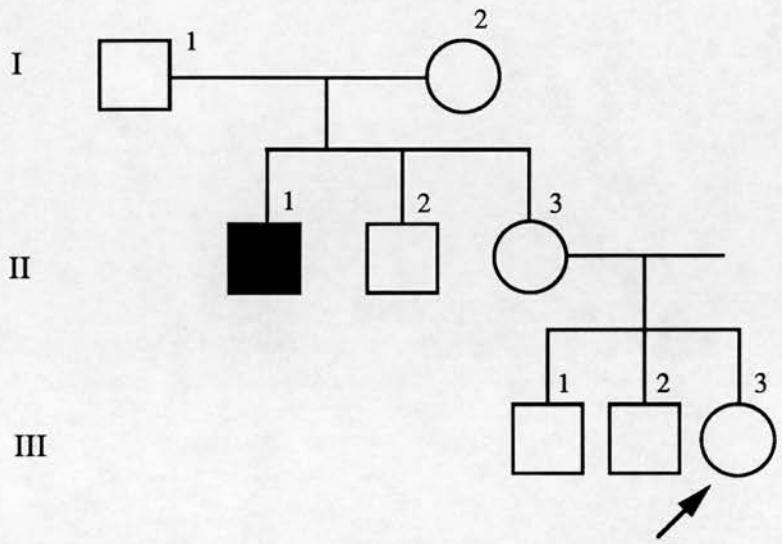
This information can then be used to advise II-3 of the risks of having an affected child. The risk that her next child will be an affected male is: 1/9 (the final risk that II-3 is a

carrier) x 1/4 (the chances of a child being both male and inheriting the abnormal X-chromosome) = 1/36. If the information provided by the three normal children is not taken into account then the risk of having an affected child is 1/2 (the risk that II-3 is a carrier from the pedigree) x 1/4 = 1/8.

The conditional probability can be further modified if any carrier tests are available. For example in the pedigree described the VIII:C/vWF:Ag ratio in II-3 was 0.65. From previous analyses of the VIII:C/vWF:Ag ratio in both obligatory carriers of haemophilia A and normal women, 3/4 obligate carriers were found to have a ratio equal to or below 0.65 but only 1/16 normal women. These results are shown in the table below and illustrate that the addition of this extra information increases the risks that II-3 is carrier and reduces the probability that she is normal.

Probability	**II-3 carrier**	**II-3 not a carrier**
Prior Probability	1/2	1/2
Conditional Probability		
- of 3 normal sons	1/8	1
- of VIII:C/vWF:Ag ratio		
= 0.65	3/4	1/16
Joint Probability	1/2 x 1/8 x 3/4 = 3/64	1/2 x 1 x 1/16 = 1/32
Final Risk or Posterior probability	3/5	2/5

Bayesian analysis can also be applied to pedigrees in which only an isolated affected male is present who may or may not be a new mutant.



In the Pedigree shown above, there is single haemophilic male (II-1) and III-3 seeks advice as to her carrier status.

The prior probability for III-3 is quite different to the prior probability established for II-3 in the previous Pedigree.

The probability that any female is a carrier can be derived from the formula:

$$\text{Probability} = \frac{2\mu + 2v + 2\mu f}{1 - f}$$

where μ is the mutation rate in females, v is the mutation rate in males and f is the biological fitness.[323] Biological fitness is the capacity to reproduce and have healthy children. In some sex-linked recessive disorders eg. Duchenne Muscular Dystrophy the biological fitness approaches or equals zero but in other conditions such as haemophilia, fitness is reduced but above zero. In conditions in which biological fitness is only mildly impaired this will have a major influence when estimating the prior probability that any female is a carrier as she may have inherited the abnormal gene from her father.

In haemophilia the biological fitness of affected males is estimated to lie between 0.3 -

0.7. The mutation rate in males (v) is thought to be about 10 times that in females (μ).[324] If we assume for the pedigree above that f=0.5 and $v = 10\mu$ then the prior probability of female being a carrier is:

$$\frac{2u+20u+2u0.5}{1-0.5}$$
$$=\frac{23u}{0.5}$$
$$=46u$$

In order to derive the risks for III-3 the risks of I-2 and II-3 being carriers must be calculated. The Bayesian table below calculates the risks for I-2:

Probability	**I-2 carrier**		**I-2 not a carrier**	
Prior Probability	46μ		1-46μ (~1)	
Conditional Probability				
1 affected son	1/2		μ	
1 unaffected son	1/2		1	
II-3	**Carrier**	**Not carrier**	**Carrier**	**Not carrier**
Prior Probability	1/2	1/2	$\mu+v = 11\mu$	1
Conditional Probability	1/4	1	1/4	1
2 normal sons				
Joint Probability	46μx1/4x1/2 x1/4=46/32 = 23μ/16	46μx1/4x1/2x1 =46μ/8 = 23μ/4	1xμx1x11μx 1/4=$11\mu^2/4$ (ignored as μ^2 is almost negligible)	1xμx1x1x1=μ

The final or posterior probability that II-3 is a carrier equals:

$$\frac{\frac{23\mu}{16}}{\frac{23\mu}{16}+\frac{23\mu}{4}+\mu}=\frac{23}{131}$$

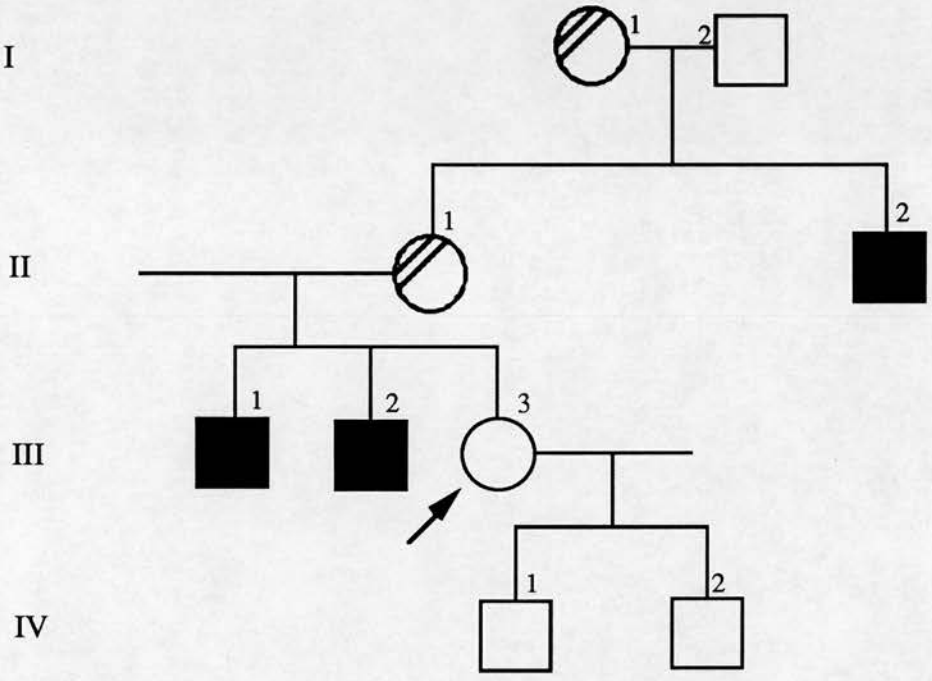
and the prior probability that III-3 is a carrier equals

$$\frac{23}{131}\times\frac{1}{2}=\frac{23}{262}$$

Additional information from, for example coagulation testing might alter these probabilities quite dramatically.

Bayesian analysis allows many variables to be included in the final risk analysis eg, coagulation data, other biochemical data eg. G6PD isoelectric focusing results, and the results of RFLP analysis.

In the following pedigree, RFLP analysis using a linked polymorphism was available.



II-1 is an obligate carrier of haemophilia A as she has a brother who is a haemophiliac and she also has two affected sons. Her daughter III-3 wished to know if she is a carrier. Genotypic analysis was carried out using a single RFLP and showed that she (III-3) had inherited the same allele as all three affected haemophiliacs and was, therefore, highly likely to be a carrier. Although this RFLP is closely linked to the FVIII gene there is a chance of a recombination occurring. The recombination fraction (θ) is an estimate of the likelihood of recombination taking place.

Therefore, if we construct a table as before:

Probability	III-3 carrier	III-3 not a carrier
Prior Probability	1/2	1/2
Conditional Probability		
2 normal sons	1/2 x 1/2 = 1/4	1
RFLP data	1-θ	θ
Joint Probability	1/2 x 1/4 x (1-θ)	1/2 x 1 x θ
	= (1-θ)/8	θ/2
Final Risk or Posterior probability	$\frac{\frac{(1-\theta)}{8}}{\frac{(1-\theta)}{8}+\frac{\theta}{2}} = \frac{1-\theta}{(1-\theta)+4\theta}$	$\frac{\frac{\theta}{2}}{\frac{(1-\theta)}{8}+\frac{\theta}{2}} = \frac{4\theta}{(1-\theta)+4\theta}$

where θ is the chance of a recombination event occurring.

If in this case θ is 0.05 then the chances of III-3 being a carrier are:

$$\frac{1-0.05}{(1-0.05)+(4\times 0.05)} = 0.82$$

and the chances of III-3 not being a carrier are:

$$\frac{4\times 0.05}{(1-0.05)+(4\times 0.05)} = 0.18$$

Bayesian analysis allows the risk of carriership to be determined taking into account many variables. However, as the size of the Pedigree increases and the amount of data available grows, the complexity of the analysis increases dramatically. Such tasks are, therefore, best analysed using computer programmes.

Chapter 2 - Materials and Methods.

2. Materials and Methods.

2.1. Patients.

Families studied had either one or more diagnosed haemophilic members and were, therefore, offered carrier detection as part of a genetic counselling service or alternatively, counselling was requested because of a history of haemophilia in previous generations. Families were seen either at the Birmingham Children's Hospital, The Queen Elizabeth Hospital, Birmingham or Addenbrooke's Hospital, Cambridge.

At an initial interview, an accurate family pedigree was plotted and samples taken for DNA studies and coagulation assays.

2.2. Historical Controls.

Haemophilia A: Phenotypic data on 31 normal females and 31 obligatory carriers of haemophilia A was available and shown in Table 15. Obligatory carriers and normal controls matched for age and their oral contraceptive status had previously been studied. For each women three assays for VIII:C and vWF:Ag were performed on separate occasions and the mean calculated. In the majority of cases vWF:RCo was also measured. The ratios of VIII:C/vWF:Ag and where appropriate the VIII:C/vWF:RCo were calculated. This information is used in the results section to interpret the results from phenotypic analysis on the study subjects (Chapter 3: Results).

Haemophilia B: No historical controls were available for haemophilia B. However, 15 normal females were studied to allow a comparison with results obtained from the study.

2.3. Analysis of Phenotypic Data.

Haemophilia A: the ratios for VIII:C/vWF:Ag and VIII:C/vWF:RCo (where possible) were derived for normal females and obligatory carriers within the control group. In women in whom both results were available the VIII:C/vWF:Ag ratio was plotted against the VIII:C/vWF:RCo ratio (Chapter 3: Results). From this graph a 'cut-off' value for each ratio was found which correctly classified all of the normal women. These ratios were then used to analyse the data derived from the phenotypic studies of both the study obligatory and potential carriers. In the obligate carriers, the number correctly classified from these ratios was determined whilst for the potential carriers, a prediction was made based, on the ratios, as to whether they were likely to be carriers or normal.

In some of the study obligatory and potential carriers only a single ratio could be calculated. In these cases, although they are not presented graphically, the original data has been reanalysed and a prediction of carriership made. These graphs and tables are reported in Chapter 3.

Haemophilia B: only limited phenotypic data was available on families with haemophilia B. The measurement of IX:C was used to predict carrier status where possible and IX:Ag assays were used to establish CRM status.

In the analysis of the data reference is made to 'unrelated females' when reporting the frequency of heterozygotes. This was performed by alphabetically sorting each family and recording the results obtained for the first female member.

Table 15. Summary of VIII:C, vWF:Ag, vWF:RCo, VIII:C/vWF:Ag and VIII:C/vWF:RCo values in 31 normal women and 31 obligatory carriers of haemophilia A used as a control group in this study.

	VIII:C (iu/dl)	vWF:Ag (iu/dl)	vWF:RCo (iu/dl)	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo
Normal women					
On oral contraceptive pill (n=13)					
No.	13	13	9	13	9
Mean	1.10	1.17	1.12	0.98	1.21
Max.	2.22	2.06	1.93	1.26	2.25
Min.	0.62	0.54	0.48	0.59	0.50
SD	0.42	0.48	0.64	0.34	0.71
Not on oral contraceptive pill (n=18)					
No.	18	18	10	18	10
Mean	1.14	1.13	1.23	1.06	1.03
Max.	1.68	2.18	2.00	1.38	2.13
Min.	0.67	0.65	0.55	0.60	0.62
SD	0.46	0.49	0.62	0.26	0.44
Obligatory carriers of haemophilia A					
On oral contraceptive pill (n=13)					
No.	13	13	11	13	11
Mean	0.52	1.02	1.02	0.54	0.66
Max	1.04	2.21	1.61	0.80	0.79
Min	0.17	0.42	0.82	0.36	0.28
SD	0.26	0.55	0.53	0.20	0.39
Not on oral contraceptive pill (n=18)					
No.	18	18	12	18	12
Mean	0.64	1.24	1.01	0.54	0.84
Max.	0.93	2.06	1.69	0.71	1.82
Min.	0.27	0.64	0.33	0.21	0.26
SD	0.23	0.39	0.51	0.20	0.52

NB. No. - number analysed; Mean - the mean of three samples collected on separate occasions; Max - maximum assay value; Min - minimum assay value; SD - standard deviation.

2.4. Reagents.

All reagents unless otherwise stated were obtained from either Sigma Chemical Co., Poole, Dorset or BDH Chemical Ltd., Bristol. All reagents were Analar grade or equivalent.

DNA Reagents.

Proteinase K, lysozyme, RNAse, bovine serum albumin (BSA), large fragment DNA polymerase I (Klenow) and dNTP's were obtained from BCL, Lewes. Restriction endonucleases, lambda DNA/Hind III and ∅X174/Hae III markers, were obtained from Gibco-BRL, Paisley Scotland. Hybond-N membrane and [^{32}P]dCTP were from Amersham International, Amersham and Saran Wrap from Du Pont (UK) Ltd., Stevenage. Hybridisation bags were obtained from Hybaid Ltd, Teddington, Middx. Hexaribonucleotides and Sephadex G50 (DNA Grade) were from Pharmacia-LKB, Milton Keynes. Fuji RX X-ray film and X-ray cassettes were obtained from Genetic Research Instrumentation Ltd., Dunmow, Essex. Whatman 3M chromatography paper was obtained from Whatman International Ltd., Maidstone, Kent. *Thermus aquaticus* 'Taq' polymerase was obtained from Perkin-Elmer Cetus.

Coagulation reagents.

Rabbit anti-human factor VIII and FIX, peroxidase anti-FVIII and anti-FIX conjugates were obtained from DAKO Ltd., High Wycombe, Bucks. Coagulation standards were obtained from The National Institute for Biological Standards and Controls (NIBSC), London. Other reference and factor depleted plasma were obtained from Immuno Ltd., Sevenoaks, Kent.

Buffers.

All buffers referred to in the methods section are listed at the end of this chapter.

2.5. Coagulation Methods.

2.5.1. Collection and Preparation of Samples for Coagulation Assays.

Samples for coagulation studies were collected into 3.8% trisodium citrate in a ratio 1:9 anticoagulant to blood. Platelet poor plasma (PPP) was prepared by centrifuging whole blood samples at 1500g for 15 minutes at 4^{o} C and removing the plasma. Functional VIII:C assays were performed immediately; for assays of vWF and FIX, samples were frozen and stored at -50^{o} C for processing in batches at a later date.

2.5.2. Two-stage Factor VIII:C Assay.

A modified commercial kit (Diagen Kit, Diagnostic Reagents Ltd.) based on a 2-stage factor assay was used for VIII:C assays.

Reagents:

1. Test plasma: platelet poor plasma prepared as above.
2. Standard: (NIBSC) reference plasma for VIII:C reconstituted to give 1.00iu/ml, stored at -20^{o} C until required and warmed to 37^{o} C for 10 minutes prior to use.
3. Factor VIII reagent: reconstituted with 5ml of isotonic saline and 5ml of 0.025M $CaCl_2$.
4. Substrate plasma: reconstituted in 5ml distilled water and kept at 37^{o} C whilst performing the assay.
5. Citrate saline: 1 part 3.8% trisodium citrate + 4 parts isotonic saline.
6. Aluminium hydroxide: 1g $Al(OH)_3$ in 4ml distilled water.

Method:

A) Adsorbed plasma: 200µl of plasma was mixed with 20µl of $Al(OH)_3$ and incubated at 37° C for 3 minutes, spun in a microfuge for 2 minutes at 12,000 rpm and the supernatant removed.

B) Plasma dilutions: Using 3" x 1/2" plastic tubes, dilutions (1/10, 1/20, 1/50, 1/100, 1/400) of standard and test plasma were prepared in citrate-saline buffer and stored on ice until required.

C) Incubation: Sufficient glass clotting tubes required for the assay were placed in an ice-block and 400µl of the factor VIII reagent added to each tube. Tubes were placed in the water bath at 37° C, allowed to warm, 100µl of the first standard dilution added and the master clock started. At 1 minute intervals, 100µl samples of the remaining standard and test dilutions were added to the prewarmed glass clotting tubes. Finally, 100µl of citrate-saline was added to the last tube as a blank.

D) Clotting: At 12 minutes on the master clock, 200µl of substrate plasma was added to the first tube and the clotting time recorded. At 1 minute intervals this was repeated with the remaining tubes.

E) Results: Clotting times were plotted against plasma dilutions on double logarithmic scale graph paper and two best-fit parallel lines drawn through each set of points. From this the VIII:C concentration in the test plasma could be determined.

2.5.3. Immunological Assay of vWF (vWF:Ag) by ELISA.

An 'in-house' ELISA method[325] was used for vWF:Ag assays.

Reagents:

1. DAKO rabbit anti-human FVIII.
2. DAKO peroxidase anti-FVIII conjugate.
3. Coating buffer - 0.05M carbonate buffer pH9.6.
4. Incubation buffer - PBS + 0.1% Tween 20.
5. Washing buffer - PBS + 0.05% Tween 20.
6. Citrate phosphate buffer (CPB) pH5.0.
7. Substrate solution - 80mg of 1,2 orthophenylenediamine dihydrochloride (OPD) + 15ml of CPB + 10µl of fresh H_2O_2 (20 volumes) prepared immediately before use.
8. NIBSC human vWF:Ag standard.
9. Normal control plasma.

Method:

A) Plate coating: 100µl of anti-human FVIII (diluted 1/500 with carbonate buffer) was added to each well of a 96 well microtitre plate using a multi-channel pipette and the plate incubated for 1 hour at 20^{o} C. The plate was then inverted to remove the antibody and washed three times in 0.05% washing solution.

B) Antigen Incubation:

I. Standards, tests and control plasma samples were diluted 1/10, 1/20, 1/40 and 1/80 in incubation buffer.

II. 100µl of buffer alone was added to the first column of the plate to serve as a negative control and 100µl of each dilution of standard, control and tests in duplicate to the remaining wells.

III. The plate was then incubated in a moist chamber for 1 hour at 20^{o} C then washed as above.

C) Conjugate Incubation: Peroxidase anti-FVIII conjugate was diluted 1/500 with incubation buffer and 100µl added to each well using a multi-channel pipette. The plate was incubated for a further 1 hour at 20° C, inverted to remove the conjugate and washed twice with washing solution and once with CPB.

D) Substrate Incubation: The substrate was prepared immediately before use and 100µl added to each well and the plate incubated on the bench. Periodically the microtitre reader was blanked using the blank wells and the OD_{492} of the 1/10 dilution checked. When the OD reading reached 0.2-0.22, the reactions were stopped by adding 150µl of 1M sulphuric acid to each well.

E) Results: The OD_{492} reading for each well was plotted on a linear scale against the standard dilution on a logarithmic scale. A standard curve was plotted and a parallel line drawn against it for each test. The value for each test sample could then be derived.

2.5.4. Measurement of von Willebrand Factor (vWF) using a 2-site Monoclonal Antibody ELISA.

vWF activity was measured using a 2-site monoclonal antibody ELISA assay.[100] This assay has been shown to detect the presence of an epitope on the vWF molecule that reflects its function.[100,326]

Reagents:

1. Primary antibody: RFF-VIII:R/1 - diluted in coating buffer.
2. Secondary antibody: RFF-VIII:R/2- alkaline phosphatase conjugate - diluted in dilution buffer.
3. Coating buffer - 0.05M carbonate buffer pH9.6.
4. Dilution buffer - PBS + 0.05% Tween 20 + 0.1% BSA.
5. Washing buffer - PBS + 0.1% Tween 20.
6. Substrate buffer - see buffers.
7. Substrate solution - p-nitrophenyl phosphate (1mg/ml) in substrate buffer.

8. vWF standard - NIBSC.

Method:

A) Plate coating: RFF-VIII:R/1 antibody was diluted in coating buffer to 1µg/ml and 220µl added to each well of a 96 well Nunc Immunoplate. The plate was incubated at 20° C for 1 hour and then overnight at 4° C. Before use the plate was washed three times with washing buffer.

B) Antigen preparation: Standard and test plasma samples were diluted in dilution buffer and 200µl added to duplicate wells. For the standard curve, the following dilutions were used: 0, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640. Test samples were diluted 1/10, 1/20 and 1/40. The plates were incubated for 2 hours at 20° C and then washed three times with washing buffer.

C) Conjugate Incubation: RFF-VIII:R/2-alkaline phosphatase conjugate was diluted 1/100 and 200µl was added to each well using a multi-channel pipette. The plate was incubated for a further 2 hours at 20° C then washed three times with washing solution.

D) Substrate Incubation: The substrate was prepared immediately before use and 200µl added to each well and the plate incubated on the bench. Exactly 30 minutes after adding the substrate solution, 50µl of 3M NaOH was added to each well to stop the reactions and the OD_{405} for each well read.

E) Results: The OD_{405} for each well was plotted on a linear scale against standard dilutions on a logarithmic scale. A standard curve was derived for the control and a parallel line drawn against it for each test. The value for the test could then be derived.

2.5.5. Functional FIX Assay - One Stage IX:C Assay.

Functional IX:C assays were performed using a 1-stage factor assay based on the partial thromboplastin time.[327]

Reagents:

1. Test plasma.
2. FIX reference plasma - NIBSC.
3. FIX deficient plasma - Immuno Ltd.
4. Light kaolin suspension (0.5%).
5. Platelet substitute (Bell & Alton).
6. Tris-buffered saline pH7.4.
7. 0.025M calcium chloride.

Method:

A) Plasma dilutions: Using 3" x 1/2" plastic tubes suitable dilutions (1/10, 1/40, 1/160) of standard and test plasma were prepared in Tris-buffered saline and stored on ice.

B) Incubation: 0.1ml kaolin, 0.1ml platelet substitute and 0.1ml deficient plasma were added to each of an appropriate number of glass clotting tubes and placed in a water bath at 37^{o} C.

C) Clotting: In quick succession 0.1ml of the 1/10 dilution of reference plasma was added to tube 1, 0.1ml of 1/40 dilution to tube 2 and 0.1ml of 1/160 to tube 3 and a 'master' clock started. At 3 minutes the procedure was repeated for the test plasma. At 10 minutes 0.1ml of 0.025M calcium chloride at 37^{o} C was added to each of the three reference plasma tubes and the clotting times for each determined. At 13 minutes this step was repeated for the test plasma.

D) Results: Using logarithmic-linear graph paper, the clotting reference times obtained for each dilution were plotted against the log of the corresponding plasma concentration using the 1/10 dilution as equivalent to 1.0u/ml. A straight line was then drawn through the points. The clotting times for the test plasma were then plotted and the best line

parallel to the control plasma obtained. The IX:C concentration of the test plasma could then be derived by interpolation of the line.

2.5.6. Immunological FIX (IX:Ag) Assay.

An ELISA assay was used to measure IX:Ag; the primary antibody was a rabbit anti-human IX:Ag and the secondary antibody a rabbit anti-human IX:Ag peroxidase conjugated.

Reagents:

1. Primary antibody - Rabbit anti-human IX:Ag (DAKO Ltd.).
2. Secondary antibody - Rabbit anti-human IX:Ag peroxidase conjugated (DAKO Ltd.).
3. Coating buffer - 0.05M carbonate buffer pH9.6.
4. Dilution buffer - PBS + 0.05% Tween 20 + 0.1% BSA.
5. Washing buffer - PBS + 0.05% Tween 20.
6. Citrate phosphate buffer (CPB) pH5.0.
7. Substrate solution - 30mg of 1,2 orthophenylenediamine dihydrochloride (OPD) + 10ml of CPB + 10µl of fresh H_2O_2 (20 volumes) prepared immediately before use.
8. Reference IX:Ag plasma - Immuno Ltd.
9. Normal control plasma.

Method:

A) Plate coating: The primary antibody was diluted 1/300 in carbonate buffer and 100µl added to each well of a 96 well microtitre plate leaving columns 1 and 12 empty to serve as blanks. Antibody was added to well 96 - the antigen blank. The plate was covered with parafilm and incubated overnight at 4^{o} C, the antibody was then tipped out and the plate blotted onto absorbent paper. 200µl of wash buffer was added to each well and the

plate incubated for 2 minutes. The plate was inverted to remove the buffer, drained by gently tapping on absorbent paper and the washing step repeated twice.

B) Sample Dilutions - A standard curve was derived using the dilutions shown:

Dilution	**Reference plasma**	**Diluting buffer**
100%	10µl	1.0ml
80	0.8 of 1/100 dilution	0.2ml
60	0.6 of 1/100 dilution	0.4ml
40	0.4 of 1/100 dilution	0.6ml
20	0.2 of 1/100 dilution	0.8ml

Test and control plasma were diluted 1/150 in dilution buffer. 100µl of each sample dilution was added to duplicate wells. 100µl of dilution buffer was added to well 96 (H12). The plate was covered with parafilm and incubated at 37° C for 60 minutes and the washing step carried out earlier, repeated.

C) Substrate conjugation: The secondary antibody was diluted 1/1000 in dilution buffer and 100µl added to each well using a multi-channel pipette. The plate was incubated for 2hrs at 37° C. (During this time 80mg of 1,2 orthophenylenediamine dihydrochloride (OPD) was dissolved in 10ml of CPB.) The plate was then washed twice with washing buffer and once with CPB.

D) Substrate incubation: 10µl of H_2O_2 (20 Vols) was added to the OPD solution immediately before use. 100µl of the OPD/H_2O_2 solution was added to each well and the colour allowed to develop at 37° C. When the colour had developed sufficiently (approximately 4 minutes) the reaction was stopped by adding 150µl of 1M sulphuric acid to each well.

E) Results: The plate was then read on a microtitre plate reader (492nm), a standard curve established by plotting dilution against OD_{492} on single log paper. The IX:Ag values in the test samples were then established from the standard curve.

2.6. DNA Methodology.

FVIII and FIX Gene Probes.

The FVIII and FIX gene probes used for the genotypic analyses are listed at the end of this chapter.

2.6.1. Collection of Whole Blood Samples for DNA Studies.

Whole blood was collected into 3.8% trisodium citrate (1:9 blood:anticoagulant). The DNA was either extracted immediately or the samples stored at -30^{o} C for processing at a later date.

2.6.2. DNA Extraction.

The method of Bell et al[328] was used with modifications. 10ml of anticoagulated blood was added to 90ml of ice-cold cell lysis buffer and stored on ice for 15 minutes. The sample was centrifuged at 1000g for 10 minutes at 4^{o} C to obtain a pellet which was then re-suspended in 4.5ml of TE pH8.0. The nuclei were lysed by adding 10ml of nuclear lysis buffer and gently rotating the sample on a mechanical rotator at 250rpm until a clear viscous solution was obtained. This was then extracted with 5ml of TE (pH8.0) saturated phenol followed by 5ml of chloroform:isoamyl alcohol (24:1) and the phases separated by centrifugation at 1000g for 5 minutes at 20^{o} C. The upper phase was re-extracted with 5ml of phenol/chloroform followed by a single chloroform extraction. The DNA was precipitated by adding 2 1/2 volumes of ethanol, the DNA collected on a sealed sterile glass Pasteur pipette, washed in 70% ethanol, dried and finally re-suspended in 500µl of distilled water.

2.6.3. Quantitation of Genomic and Plasmid DNA.

Genomic and plasmid DNA was quantitated by measuring its optical density (OD) at 260nm. The degree of phenol and protein contamination was determined by measuring

the OD_{260} and OD_{280}.

From the formula:

1 OD_{260nm} = 50µg double stranded DNA - the DNA concentration could be determined.

The ratio of the OD's at 260/280 also gives an indication of DNA purity (should exceed 1.8).

2.6.4. Restriction Enzyme Digests.

Restriction enzyme digests were performed according to the manufacturer's instructions supplied with each enzyme. 10µg of DNA was used in each digest, 2units/µg of enzyme and the appropriate volume of a 10X buffer. Samples were digested for at least 2 hours and usually overnight (12-16 hours).

2.6.5. Submarine Gel Electrophoresis.

Following restriction enzyme digestion, DNA samples were size fractionated by submarine gel electrophoresis in 1X Tris-Borate-EDTA (TBE). 20 x 17cm agarose gels (0.8%) in 1X TBE containing ethidium bromide (0.5µg/ml) were poured and allowed to set. Sufficient 1X TBE buffer to cover the gel by 2-3mm was added. One-sixth volume of 6X loading buffer was added to each DNA sample and the samples loaded using a micropipette. Up to twenty samples could be run on a single gel. Electrophoresis was carried out at a constant voltage of 1.5V/cm until the bromophenol blue front had migrated to the end of the gel.

Size markers (1µg of Lambda DNA digested with Hind III) were included in each electrophoresis from which the sizes of individual fragments could be determined (the migration of a fragment being inversely proportional to its molecular weight). In addition by incorporating radio-labelled markers, the efficiency of transfer could be

monitored and markers were then easily visible on the final autoradiograph.

2.6.6. Preparation of ^{32}P-Labelled Molecular Weight Markers.

The large fragment of DNA polymerase I (Klenow) was used to radio-label the recessed 3' ends of Lambda DNA digested with Hind III.

The following were combined:

Lambda DNA/Hind III fragments	1µg
10X labelling buffer	2.5µl
Unlabelled dNTP's (minus dCTP)	2 nmoles of each
[^{32}P]dCTP (sp. act >400Ci/mmol)	2 pmoles
Klenow DNA Polymerase	1 unit
Distilled water	to 25µl

and incubated at 20^{o} C for 30 minutes. The reaction was stopped by the adding 1µl of 0.5M EDTA (pH8.0) and the unincorporated [^{32}P]dCTP removed by Sephadex G50 column chromatography (see 2.6.17).[329] Labelled markers were stored at -20^{o} C and could be used for several months before losing activity.

2.6.7. Southern (Capillary) Blotting.

DNA fragments following separation by electrophoresis were transferred to nylon membranes by capillary blotting.[134] After electrophoresis the gel was placed in denaturing solution for 15 minutes to produce single-stranded DNA. This was repeated twice leaving the final solution for 30 minutes. Denaturing buffer was replaced with neutralising buffer left for 30 minutes and repeated twice. If fragments greater than 20kb were to be transferred then the gel was placed in 0.25M HCl at room temperature for 15 minutes prior to denaturation to hydrolyse the fragments and improve the transfer.

The transfer apparatus consisted of a glass plate suspended over a reservoir of transfer

buffer (10X SSC). A sheet of filter paper (Whatman 3M) was cut to the same width as the gel but long enough to form a wick from the glass plate into the buffer. The gel was placed onto the filter paper and all air bubbles excluded. A sheet of nylon membrane (Hybond N) previously cut to fit the gel, was placed on the gel and all air bubbles excluded. Three sheets of filter paper were then cut slightly larger than the gel, soaked in transfer buffer and placed on top of the membrane followed by a stack of absorbent paper towels. A glass plate and a 0.5kg weight were used to compress the stack. Transfer with 10X SSC was allowed to proceed for 12-16 hours, after which the membrane was removed, rinsed briefly in 2X SSC to remove any adhering agarose and allowed to dry at room temperature. The membrane was then wrapped in 'Saran Wrap' and the DNA immobilised by exposing it to UV light (305nM) for 3 minutes.

2.6.8. Pre-Hybridisation and Hybridisation of Southern Blots.

Detection of specific gene sequences in immobilised DNA involved an initial pre-hybridisation step to block non-specific binding of radio-labelled probe during subsequent hybridisation. This was then followed by the actual hybridisation with the radio-labelled probe.

Membranes were heat sealed into plastic bags (Hybaid Ltd.) with sealable inlet/outlet ports. 25ml of buffer was used in each pre-hybridisation/hybridisation and consisted of:

Solution	**Volume**	**Final Concentration**
20X SSC	7.5ml	SSC X6
Denhardt's solution 50X	2.5ml	X5
10% SDS	1.25ml	0.5%
Dextran Sulphate 25%	5ml	10%
Distilled water	8.75ml	

Denatured Salmon Sperm (10mg/ml)	200µl	0.08mg/ml
Final volume:	25ml	

Salmon sperm was used as a source of non-homologous DNA to reduce non-specific binding of the radio-labelled probe and was denatured by boiling for 5 minutes followed by rapid chilling on ice. Pre-hybridisation was carried out with shaking at 65° C for 6-7 hours after which the pre-hybridisation buffer was drained, added to the labelled denatured probe and readded to the membrane ensuring all air bubbles were expelled. Hybridisation was carried out with constant shaking for 24-48 hours at 65° C.

2.6.9. Washing of Hybridisation Membranes (Southern Blots).

Following hybridisation membranes were washed to remove any non-hybridised probe. Three washes were used with increasing temperature and stringency:

1. SSC X2 + 0.1% SDS at 20° C for 15 minutes
2. SSC X2 + 0.1% SDS at 65° C for 15 minutes
3. SSC X0.2 + 0.1% SDS at 65° C for 30 minutes

Filters were blotted to remove excess fluid but not allowed to dry and wrapped in Saran Wrap. Autoradiography was carried out for 1-5 days at -70° C using Fuji RX film, Kodak cassettes and two intensifying screens.

2.6.10. Reprobing of Hybridisation Membranes (Southern blots).

Hybridised membranes could be re-probed by stripping in denaturation buffer for 1 minute, followed by a wash in distilled water for 30 seconds and then 2 minutes in neutralisation buffer. Filters were then washed briefly in distilled water, wrapped in Saran Wrap and autoradiographed for 3 days to ensure stripping was complete. The filters were

then pre-hybridised and hybridised as before.

2.6.11. Growth, Maintenance and Preservation of Bacterial Strains.

E. coli strain DH5 was a gift from Dr. I. Peake (Dept. of Haematology, Welsh National School of Medicine, Cardiff) and was used for all transformation experiments.

2.6.11.1. Short-term Storage of Bacteria/clones.

Bacteria were stored inverted on tightly sealed agar plates at 4^{o} C until required. Colonies could be maintained for 2-4 weeks by this technique.

2.6.11.2. Long-term Storage of Bacteria/clones.

A single bacterial colony was inoculated into a culture flask containing 10ml of 2XTY (Bacto-tryptone, Bacto-yeast extract, NaCl) and grown overnight at 37^{o} C with vigorous agitation. 0.85ml of the overnight culture was then transferred to a sterile pre-chilled Eppendorf containing 0.15ml of freezing buffer, vortexed briefly, snap frozen in liquid nitrogen and stored at -70^{o} C. Bacteria were recovered by scratching the surface of the frozen stock with a sterile platinum loop and either streaking an agar plate or inoculating a flask containing the appropriate culture media.

2.6.12. Transformation of E.coli by Plasmid DNA.

The FVIII and FIX gene probes were received cloned into plasmids and required to be transfected into a suitable strain of E.coli to provide sufficient amounts for use as probes. The method used was based upon that of Mandel and Higa[330] who demonstrated that the uptake of bacteriophage Lambda is enhanced by treatment with calcium chloride. This technique was subsequently was shown to work for plasmid DNA.[331]

100ml of 2XTY was inoculated with 1ml of an overnight bacterial culture and the cells grown until they reached a density of 5 x 10^7/ml. Cell density was calculated by measuring the OD_{600} of the culture. At the correct cell density the culture was chilled on ice for 10 minutes and then centrifuged at 4000g for 5 minutes at 4° C. The supernatant was discarded, the cells resuspended in 50ml of ice cold 100mM $CaCl_2$ and stored on ice for 15 minutes. The cells were then centrifuged at 4000g for 5 minutes at 4° C, the supernatant discarded and the cells resuspended in 6ml of ice-cold 100mM $CaCl_2$. Aliquots of 0.2ml were dispensed into pre-chilled, sterile, glass tubes and the cells stored at 4° C for 24 hours. Plasmid DNA was diluted in TE (pH8.0) to a concentration of 1ng/µl and 40ng added to 0.2ml of competent cells and stored on ice for 30 minutes. The tubes were then heat shocked in a water bath at 42° C for 2 minutes, 1.0ml of 2XTY was then added to each tube and the cells incubated at 37° C for 60 minutes without shaking to allow the bacteria to recover. Each tube was then mixed with 3ml of top agar (Bacto-agar 7g/l + agarose 7g/l) and poured onto pre-heated agar plates (Bacto-agar 15g/l + agarose 15g/l) containing the appropriate selective agent (usually ampicillin 50µg/ml) and incubated at 37° C for 16-24 hours. This method yielded approximately 10^5-10^7 transformants per µg of intact plasmid.

2.6.13. Rapid, Small-scale Isolation of Plasmid DNA.

A small-scale plasmid preparation was performed following each transformation to check the insert before proceeding to a large scale preparation. A single bacterial colony was inoculated into 5ml of 2XTY (containing the appropriate selective agent eg. ampicillin 50µg/ml) and the cells grown at 37° C overnight with vigorous agitation. 1.5ml of this overnight culture was transferred to an Eppendorf and centrifuged for 60 seconds to pellet the cells. The pellet was then re-suspended in 0.35ml of:

Sucrose	8%
Triton X-100	0.5%

EDTA pH8.0	50mM
Tris-HCl pH8.0	10mM

containing lysozyme (10mg/ml in 10mM Tris-HCl pH8.0) and the cells vortexed to mix. The tube was placed in a boiling water bath for 40 seconds and then centrifuged for 10 minutes at room temperature at 12,000rpm in a microfuge. The pellet was removed, 40µl of 2.5M sodium acetate and 420µl of isopropanol added and the sample placed in a dry-ice/ethanol bath for 15 minutes to precipitate the plasmid DNA. Samples were centrifuged again for 15 minutes at 4° C, the supernatant removed and the plasmid pellet resuspended in 50µl of TE pH8.0 containing DNAse free RNAse (50µg/ml) and incubated at 37° C for 10 minutes. 10µl of the solution was then added to 1.2µl of the appropriate 10X buffer and digested with 1-2 units of the desired restriction enzyme. Samples were analysed by agarose gel electrophoresis in a 'minigel' system and fragments visualised on a UV transilluminator. When a 'mini-prep' demonstrated that the correct sized insert was present then a large scale isolation of the plasmid was performed.

2.6.14. Large Scale Isolation of Plasmid DNA.

500ml of 2XTY in a 2 litre flask was inoculated with a single bacterial colony containing the appropriate plasmid and grown overnight at 37° C with vigorous agitation. The cells were pelleted by centrifugation at 4000g for 10 minutes at 4° C, re-suspended in 3ml of glucose buffer containing lysozyme (20mg/ml in glucose buffer) and stored on ice for 30 minutes. 4ml of 0.2M NaOH/1% SDS (freshly made) was added and the mixture inverted gently to mix. The sample was stored on ice for 5 minutes, 3ml of 3M NaOAc (pH4.8) added and the sample incubated on ice for 60 minutes. The sample was then spun at 3000g for 30 minutes at 4° C to pellet the cellular DNA and bacterial debris leaving the plasmid DNA in the supernatant. The supernatant was aspirated, 2.5 volumes of absolute alcohol added and the sample placed at -70° C for 30 minutes to allow the plasmid DNA

to precipitate. The sample was spun at 10,000g for 30 minutes at 20° C to pellet the plasmid DNA. The plasmid DNA was re-suspended in 400µl of NEST buffer, transferred to a 1.5ml Eppendorf, 100µl of proteinase K (10mg/ml) and 100µl of 10% SDS added, and the sample incubated at 55° C for 60 minutes. The DNA was extracted once with phenol and once with chloroform and the DNA re-precipitated with 2.5 volumes of absolute alcohol and 1/10 volume of 3M NaOAc pH4.8 and left at -70° C for 30 minutes. The DNA was pelleted by spinning at 12,000rpm in a microfuge for 10 minutes at 4° C, re-suspended in 380 µl of distilled water, 20µl of DNAse free RNAse (10mg/ml in TE) added and the sample incubated at 37° C for 30 minutes. The DNA was precipitated by adding 2.5 volumes of ethanol and 1/10 volume of 4M NaOAc and left at -20° C for 10 minutes. The DNA was pelleted in a microfuge for 10 minutes, washed in 70% ethanol and finally re-suspended in 100-200µl of TE (pH8.0) and the concentration determined.

2.6.15. Gel Purification of Plasmid Inserts.

Following the preparation of plasmid DNA, the insert was excised from the vector by digestion with the appropriate restriction enzymes(s) (see Probe Data) and separated by electrophoresis in 1% low melting point (LMP) agarose in 1X TAE (Tris-Acetate-EDTA) containing ethidium bromide (0.5µg/ml). The band of interest was visualised under UV light and excised.

2.6.16. Labelling of Probes with [^{32}P]dCTP.

Probes were radio-labelled with ^{32}P-dCTP using the method of Feinberg and Vogelstein.[332,333] The desired band was cleanly excised from LMP agarose and placed into a pre-weighed 1.5ml Eppendorf tube. Water was added at a ratio of 3ml H_2O/gram of gel. The tube was boiled for 7 minutes to melt the gel and denature the DNA aliquoted into 20µl samples and stored at -20° C. Prior to labelling the tube was reboiled

for 3 minutes and then incubated at 37^{o} C for 10-60 minutes. Samples were re-boiled a maximum of three times. The concentration of the probe could be calculated knowing the amount of plasmid plus probe which was initially digested together with the sizes of both the vector and insert. The labelling reaction was carried out at room temperature by adding the following reagents:

Oligo-labelling buffer (OLB)	10µl
BSA 20mg/ml	1µl
DNA in agarose (maximum 33.5µl)	Xµl
[^{32}P]dCTP (Amersham PB10205) 4000Ci/mmol)	5µl
Klenow DNA polymerase (1unit/µl)	2µl
dH_2O to:	50µl

Generally, 20-30ng of DNA was used in each labelling reaction and the incubation carried out overnight. The unincorporated [^{32}P]dCTP was separated from the labelled product on a 5ml Sephadex G50 column.

2.6.17. Sephadex G50 Chromatography.[329]

A 5ml disposable plastic pipette was filled with Sephadex G50 (Pharmacia-LKB Ltd.) pre-swollen with TE pH8.0. The column was washed several times with TE (pH8.0), the labelled probe applied in a 100µl volume and the column further washed with TE. By closely monitoring the column with a mini-monitor two radioactive peaks could be detected. The first peak (representing the labelled probe) was collected whilst the second (the unincorporated [^{32}P]dCTP) was discarded.

2.6.18. Polymerase Chain Reaction (PCR).
2.6.18. Enzymatic Amplification of the Bcl I and Xba I Polymorphisms.

Using the polymerase chain reaction (PCR)[240] the regions flanking the Bcl I and Xba I polymorphisms were amplified, digested with the restriction enzymes Bcl I and Xba I respectively and RFLP analysis performed.

The primer sequences used in the PCR amplifications were as published by Kogan et al.[202]

Bcl I: 5'- TAAAAGCTTTAAATGGTCTAGGC and

5'- TTCGAATTCTGAAATTATCTTGTTC

Xba I: 5'- CACGAGCTCTCCATCTGAACATG and

5'- GGGCTGCAGGGGGGGGGGACAACAG

Amplification primers were synthesised on an Applied Biosystems synthesiser and were not purified before use. Amplifications were performed in 100µl reaction volumes containing 1µg of genomic DNA, 100pmoles of each amplification primer, 200µM of each dNTP (previously adjusted to pH7.0 with 0.05M Tris base in distilled water) and 10µl of 10X PCR buffer. Samples were heated to 100^{o} C for 5 minutes to denature the DNA, briefly spun and placed in a programmable heating block (Hybaid Ltd., Intelligent Heating Block) at the appropriate annealing temperature for 2 minutes. Two units of *Thermus aquaticus* ('Taq' polymerase) was added to each sample and the tubes overlaid with 100µl of light mineral oil.

For the Bcl I polymorphism, samples were subjected to 35 cycles in a programmable heating block (Intelligent Heating Block, Hybaid Ltd, Middx.) consisting of a 65^{o} C annealing step for 1 second, a 76^{o} C extension step for 15 seconds and a 94^{o} C denaturation step for 1 second. On the final cycle the extension step was increased to 10 minutes. The Xba I amplifications were identical but the extension time was reduced to 10 seconds. The timing for each temperature represents the time at that particular

temperature.

After the amplifications were completed, 18µl of each reaction was analysed on a 1.5% agarose gel containing ethidium bromide (0.5µg/ml) to check the quality of the amplified product. If a satisfactory amplification was achieved, 18µl of the amplified material was digested with 5 units of the appropriate enzyme. The samples were incubated at either 55^{o} C (Bcl I) or 37^{o} C (Xba I) for 4 hours and the digests analysed by electrophoresis. The Bcl I digests were analysed on 1.5% agarose gels containing ethidium bromide (0.5µg/ml) whilst the Xba I amplifications required electrophoresis in 12% nondenaturing acrylamide gels followed by immersion for 15 minutes in 1X TBE containing ethidium bromide 0.5µg/ml. The gels were then viewed under UV light and photographed.

2.7. Table 16. Buffers.

Aluminium hydroxide:	25g of aluminium hydroxide was dissolved in 100ml distilled water and stored in aliquots at 4^{o} C	
Carbonate buffer (0.05M):	Sodium carbonate	1.59g
	Sodium bicarbonate	2.93g
	Sodium azide	0.2g
	Distilled water to 1L (final pH9.6)	
Cell lysis buffer (CLB):	Sucrose (Analar)	110g
	Tris-HCl 1M pH8.0	10mls
	$MgCl_2$ 1M	5mls
	Triton X-100	10mls
	Made up to 1L with distilled water and stored at 4^{o} C.	
Citrate-saline:	1:4 3.8% trisodium citrate:isotonic saline	
Citrate phosphate buffer:	Citric acid	7.3g
(CPB)	Na_2HPO_4. $12H_2O$	23.87g
	Distilled water to 1L (final pH 5.0)	
Denaturation buffer:	Sodium chloride	1.5M
	Sodium hydroxide	0.5M
Denhardt's solution:	Bovine serum albumin (BSA)	2% (w/v)
	Ficoll 400	2% (w/v)
	Polyvinyl pyrollidone (PVP)	2% (w/v)
Dilution buffer:	Phosphate buffered saline (PBS)	1L
	Tween 20	0.5ml
	BSA	1g
Freezing buffer (pH6.2):	Potassium chloride	100mM
	Calcium chloride dihydrate	50mM
	Glycerol	10% (w/v)
	Potassium acetate	10mM

Gel loading buffer (X6):	Sucrose	40% (w/v)
	Bromophenol blue	0.25% (w/v)
	Xylene cyanole	0.25% (w/v)
Glucose buffer:	Glucose	10mM
	Tris-HCl pH8.0	25mM
	Na_2EDTA	10mM
Kaolin suspension (0.5%):	0.5g of light Kaolin was suspended in 100ml 0.9% saline and stored at 4° C.	
Lambda labelling buffer:	Tris-HCl pH7.2	0.5M
	Magnesium sulphate	0.1M
	Dithiothreitol	1mM
	BSA	500µg/ml
NEST buffer:	Sodium acetate	100mM
	Na_2EDTA	1mM
	SDS	1%
	Tris-HCl pH8.0	40mM
	pH adjusted to 4.8 with glacial acetic acid.	
Neutralising buffer:	Sodium chloride	1.5M
	Tris-HCl pH7.2	0.5M
	Na_2EDTA	0.001M
Nuclear lysis buffer (NLB):	Lithium dodecyl sulphate (LDS)	2%
	Lithium acetate	0.32M
	Tris-HCl pH8.0	10mM
	Na_2EDTA	1mM
Phenol:	Chromatography grade phenol was melted at 68° C and one-fifth the volume of TE pH8.0 added + 8-hydroxy-quinoline (0.1% w/v). Phenol was stored at 4° C.	

Pre-hybridisation buffer:	SSC	6X
	Denhardt's solution	5X
	SDS	0.5%
	Dextran sulphate	5%
Oligo-labelling buffer:	**Solution O:**	
(OLB)	Tris-HCl pH8.0	1.25M
	Magnesium chloride	0.125M
	Solution A:	
	2-mercaptoethanol	2µl
	Solution O	1ml
	dATP, dGTP, dTTP	5µl of each
	(each dNTP dissolved in 3mM Tris-HCl pH7.0/0.2mM EDTA at a concentration of 0.1M	
	Solution B:	
	Hepes pH6.6.	2M
	Solution C: Hexaribonucleotides (Pharmacia-LKB) evenly suspended in TE at 90 OD_{260} units/ml).	
	Solutions A, B and C were mixed in a ratio 100:250:150 and stored at -20° C.	
PCR buffer (10X):	Magnesium chloride	15mM
	Potassium chloride	500mM
	Tris-HCl pH8.8	100mM
	Gelatin	200µg/ml
Phosphate buffered saline:	NaH_2PO_4. $2H_2O$	0.37g
(PBS)	Na_2HPO_4. $12H_2O$	2.68g
	Sodium chloride	8.47g
	Distilled water to 1L	

RNAse free of DNAse:	Ribonuclease A was dissolved in TE pH8.0, boiled for 15 minutes to inactivate any contaminating DNAse and stored at -20^{o} C.	
SSC 20X:	Sodium chloride	3.0M
	Trisodium citrate	0.3M
Substrate buffer:	Diethanolamine	97ml
	Distilled water	800ml
	$MgCl_2$:$6H_2O$	100mg
	Sodium azide	0.2g
	pH adjusted to 9.8 with 1M HCl. Volume made up to 1L.	
TE pH8.0:	Tris base	10mM
	Na_2EDTA	1mM
	pH adjusted to 8.0 with HCl.	
Tris-Acetate-EDTA (50X):	Tris base	242g
	Glacial acetic acid	57.1ml
	Na_2EDTA 0.5M pH8.0	100ml
	Distilled water to 1L.	
Tris-Borate-EDTA (10X):	Tris base	108g
	Boric acid	55g
	Na_2EDTA	9.3g
	Distilled water to 1L.	
Tris-buffered saline (pH7.4):	Tris base	1.6g
	Sodium chloride	2.2g
	HCl	42ml
	Distilled water to 1L.	
Washing buffer:	PBS	0.01M pH7.2
	Tween 20	0.05%

2.8. Table 17. DNA probes used for the genotypic studies in haemophilia A.

Probe	Size	Vector	Cloning site	Polymorphisms	References	Comments
Extragenic						
ST14 (DXS15)	3.0kb	pBR322	EcoR I	Taq I Msp I	Oberlé et al, 1985[191]	Genomic probe detecting a polymorphic region closely linked to the FVIII gene.
Kindly provided by Dr. J.L. Mandel, Strasbourg.						
DX13 (DXS52)	2.0kb	pAT153	EcoR I	Bgl II	Harper et al, 1984[190]	Genomic probe detecting a polymorphic region closely linked to the FVIII gene.
Kindly provided by Dr. K. Davies, Oxford.						
Intragenic						
p114.12	0.65kb	pUC12	BamH I/Sst I	Bcl I	Gitschier et al, 1985[193]	Genomic probe detecting a polymorphism in the intron 3' to exon 18.
Obtained from ATCC.						
p482.6	9.60kb	pUC8	EcoR I	Xba I	Wion et al, 1986[195]	Genomic probe detecting a polymorphism in intron 22.
Obtained from ATCC.						
Probe C	1.8kb	pSP64	EcoR I	Bgl I	Antonarakis et al, 1985[194]	cDNA probe detecting a polymorphism at the 3' end of the FVIII gene adjacent to exon 26.
Obtained from Genetics Institute.						

ATCC - American Type Culture Collection, Rockville Maryland. USA.
Genetics Institute Inc., Boston. USA.

2.9. Table 18. DNA probes used for the genotypic studies in haemophilia B (Christmas disease).

Probe	Size	Vector	Cloning site	Polymorphisms	References	Comments
Probe VIII Kindly donated by Prof. G. Brownlee, Oxford.	2.3kb	pATX	BamH I/ Hind III	Taq I	Camerino et al, 1984[120]	Genomic probe detecting a Taq I polymorphism 3' to exon D.
Probe VIII Kindly donated by Prof. G. Brownlee, Oxford.	2.3kb	pATX	BamH I/ Hind III	Xmn I	Winship et al, 1984[135]	Genomic probe detecting an Xmn I polymorphism 3' of exon C.
Probe XIII Kindly donated by Prof. G. Brownlee, Oxford.	1.4kb	pATX	BamH I/ Hind III	Dde I	Winship et al, 1984[135]	Genomic probe detecting a Dde I polymorphism 5' of exon B.

Chapter 3 - Results.

The Results of Carrier Detection Studies in Haemophilia.

Introduction.

This section presents the results of the phenotypic and genotypic analyses performed on families with haemophilia A and B. The phenotypic, genotypic and pedigree data for each family are contained in the Appendices together with an analysis of the results for each individual. The results obtained for haemophilia A and B will be presented separately. A summary of the findings for each family is presented at the end of this Chapter in Tables 34-37.

3. Section 1.

3.1.1. The Results of Carrier Detection Studies in Haemophilia A.

Forty-four families with haemophilia A were investigated (Table 19). A family history of haemophilia affecting more than one generation was obtained in 21 families, whilst in 22 families the disease appeared to have arisen spontaneously. In 3 families from this latter group (Pedigrees Nos. 7, 12 and 24), the birth of a second affected child established the mothers as obligatory carriers. In 1 family (Pedigree No. 29) with two affected offspring the mother was adopted and no family history was obtainable, although she is, by definition an obligate carrier.

The 44 families studied comprised 232 individuals, 107 were males and 125 females. Of the males, 53 were haemophiliacs and of these 37 had severe disease (VIII:C <0.01iu/dl), 7 had moderately severe disease (VIII:C>0.01 but <0.05iu/dl) and 9 had mild disease (VIII:C >0.05iu/dl). When the 125 females from these families were analysed with their respective family pedigrees, 40 were identified as obligate carriers, 80 could not be excluded as carriers ie. were potential carriers and 5 could be classified as normal females as in each case they were wives of haemophiliacs and from families in which there was no history of haemophilia.

Table 19. Numerical summary on 232 individuals from 44 families with haemophilia A.

Number of males studied.

Normal:	54		
Haemophiliac:	53	VIII:C <0.01iu/dl	37
		VIII:C >0.01<0.05iu/dl	7
		VIII:C >0.05iu/dl	9
Total:	**107**		

Number of females studied.

Normal:	5
Potential carriers:	80
Obligate carriers:	40
Total:	**125**

3.1.2. Analysis and Interpretation of the Phenotypic Data from the Haemophilia A Kindred.

3.1.2.1. VIII:C, vWF:Ag, vWF:RCo, VIII:C/vWF:Ag and VIII:C/vWF:RCo Values in Obligatory and Potential Carriers from 44 Families with Haemophilia A.

The levels of VIII:C, vWF:Ag, vWF:RCo and the VIII:C/vWF:Ag and VIII:C/vWF:RCo ratios together with their ranges, means and standard deviations for the 80 potential carriers and 40 obligate carriers identified in the study are shown in Table 20. When VIII:C, vWF:Ag and vWF:RCo data is not given it was generally because these individuals were bled at other centres and the samples sent by post for DNA analysis.

1. VIII:C Assays.

The measurement of VIII:C has been shown to be a poor discriminant for determining carriership in families with Haemophilia A. However, its measurement can identify those women with low levels of VIII:C and, therefore, at risk of bleeding and in addition allows the VIII:C/vWF:Ag and VIII:C/vWF:RCo ratios to be derived. The results of the VIII:C assays are shown in Table 20 and Figure 11.

i. Normal females: 5 women, wives of haemophilic men and without any family history of haemophilia were bled primarily for genotypic studies. All of these women had normal VIII:C, vWF:Ag and vWF:RCo results.

ii. Obligate carriers: of the 40 obligate carriers available for analysis, VIII:C assays were available on 37. The mean VIII:C was 0.60iu/dl with a range of 0.15-1.5iu/dl.

iii. Potential carriers: of the 80 potential carriers, VIII:C assays were available on 74. The mean VIII:C was 0.83iu/dl with a range of 0.16-2.05iu/dl.

Table 20. Phenotypic data for 120 females from 44 kindred with haemophilia A and for the historical controls.

Category	VIII:C (iu/dl)	vWF:Ag (iu/dl)	vWF:RCo (u/dl)	VIII:C/ vWF:Ag ratio	VIII:C/ vWF:RCo ratio
Study data.					
Potential carriers					
Number assayed (n)	74	74	59	74	56
Mean	0.83	1.00	1.09	0.83	0.76
Range	0.16-2.05	0.45-2.10	0.35-2.30	0.16-1.87	0.18-2.56
SD	0.30	0.39	0.45	0.37	0.59
Obligate carriers					
Number assayed (n)	37	38	30	37	30
Mean	0.60	1.02	1.08	0.59	0.55
Range	0.15-1.5	0.41-2.3	0.22-2.2	0.15-1.62	0.16-2.7
SD	0.33	0.45	0.50	0.34	0.53
Historical controls.					
Normal women					
Number assayed (n)	31	31	19	31	19
Mean	1.12	1.15	1.17	1.02	1.12
Range	0.62-2.22	0.54-2.18	0.48-2.00	0.59-1.38	0.50-2.25
SD	0.44	0.48	0.63	0.30	0.58
Obligate carriers					
Number assayed (n)	31	31	23	31	23
Mean	0.57	1.13	1.02	0.54	0.75
Range	0.17-1.04	0.42-2.21	0.33-1.69	0.21-0.80	0.26-1.82
SD	0.25	0.47	0.52	0.20	0.45

NB. Mean values shown for the study group are based on a single estimation but for the historical controls represent the mean of three separate samples collected on different occasions.
SD - standard deviation.

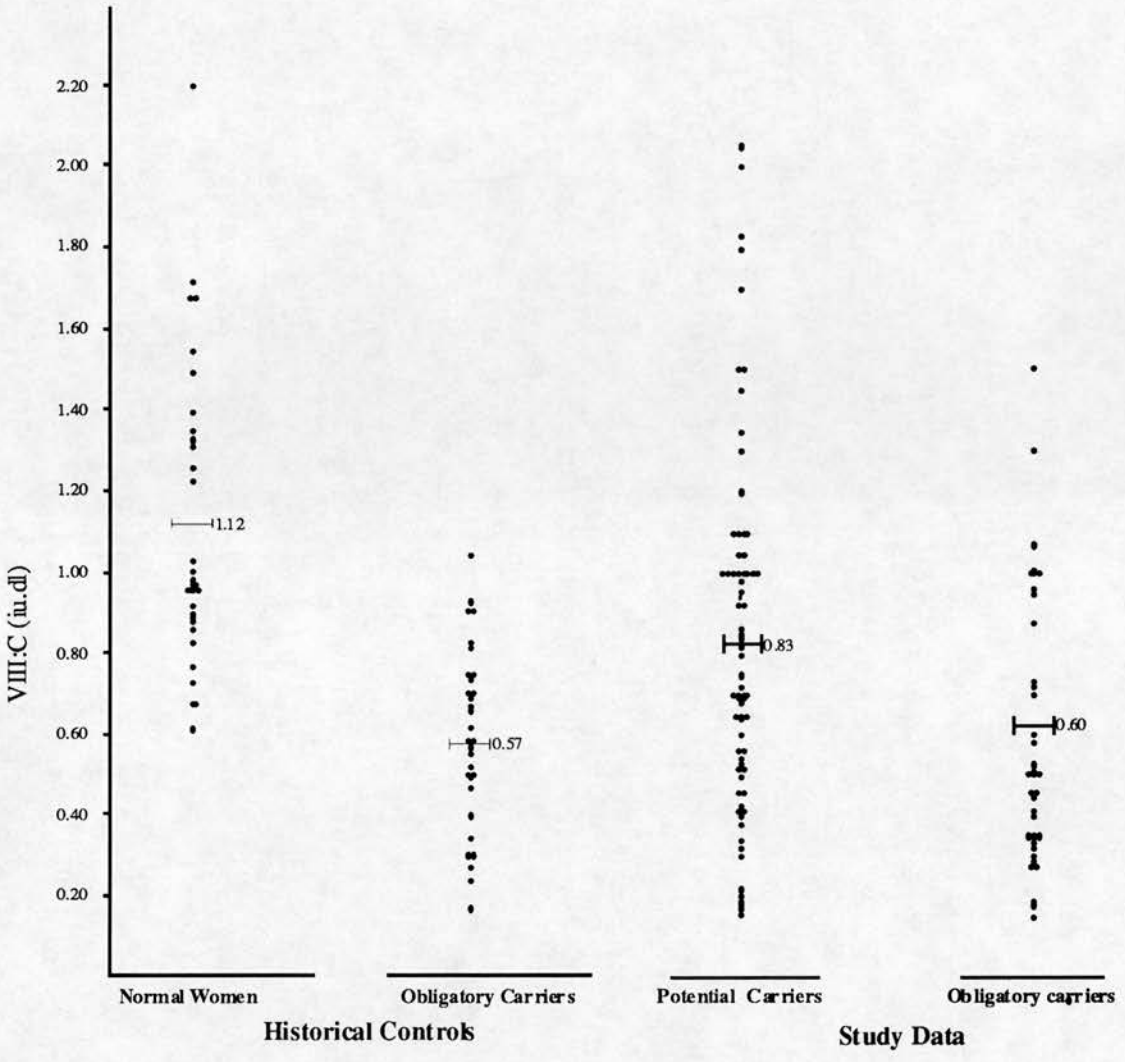


Figure 11. Scattergram showing the distribution of VIII:C in the study obligatory carriers and potential carriers of haemophilia A compared to the historical controls (normal women and obligatory carriers). The mean value for each group is indicated.

The results show that the mean VIII:C for the historical obligatory carriers and the obligatory carriers identified within the study are similar and lower than the mean VIII:C for the normal historical female controls, whilst the mean for the potential carriers lies between these two groups.

2. Assessment of Haemostatic Risk in Females from Families with Haemophilia A using VIII:C Assays.

The incidence of VIII:C values below 0.40iu/dl was 7/31 in the historical obligatory carrier group, 13/37 in the study obligate carriers and 11/74 in the study potential carriers. Except when VIII:C assays are very low, this information if of little help in carrier detection but is important as females with low VIII:C levels may have abnormal bleeding in association with invasive procedures including those used for antenatal diagnosis.

3. vWF:Ag, vWF:RCo Assays and VIII:C/vWF:Ag and VIII:C/vWF:RCo ratios.

The ratios of VIII:C to vWF:Ag and possibly VIII:C to vWF:RCo have been shown to be better discriminants of carriership than measurements of VIII:C alone. These ratios have, therefore, been calculated for the potential carriers and obligatory carriers identified within the study and compared to the mean values for the historical normal and obligatory carrier control groups. The results are shown in Table 20 and Figures 12 and 13.

A. VIII:C/vWF:Ag.

The values obtained from the measurement of the VIII:C/vWF:Ag ratio in the control and study obligatory carriers are similar although the range is larger in the latter group. The mean ratios of both groups of obligatory carriers are lower than those for the normal female control group. The mean ratio for the potential carriers lies between that of the normal women and the obligatory carrier groups.

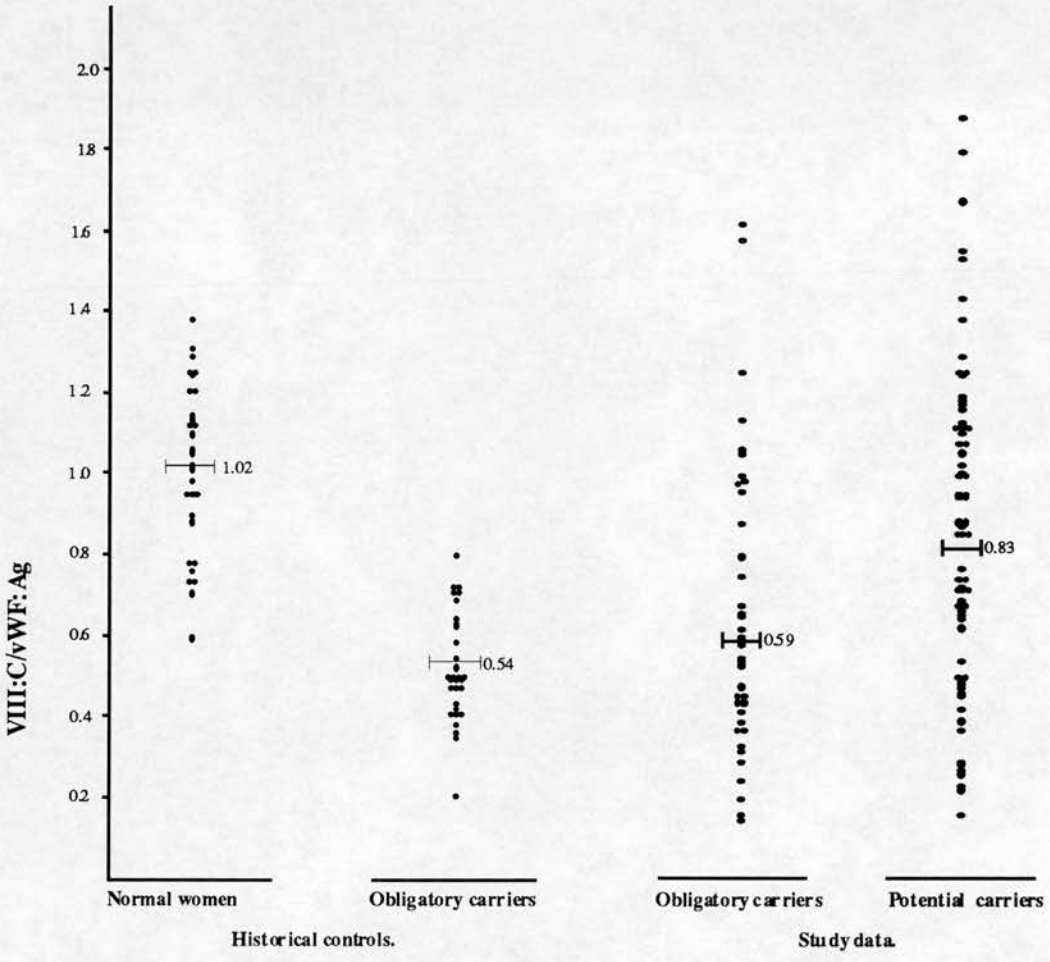


Figure 12. Scattergram showing the distribution of the VIII:C/vWF:Ag ratio in the study obligatory carriers and potential carriers of haemophilia A compared to the historical controls (normal women and obligatory carriers). The mean value for each group is indicated.

B. VIII:C/vWF:RCo.

The mean values obtained from the measurement of the VIII:C/vWF:RCo ratio in the control obligatory carriers and the study obligatory carriers are again similar but the range is much wider than that seen with the VIII:C/vWF:Ag ratio. The means are again lower than those seen for the normal female controls. The mean ratio for the potential carriers lies between that of the normal women and the obligatory carriers.

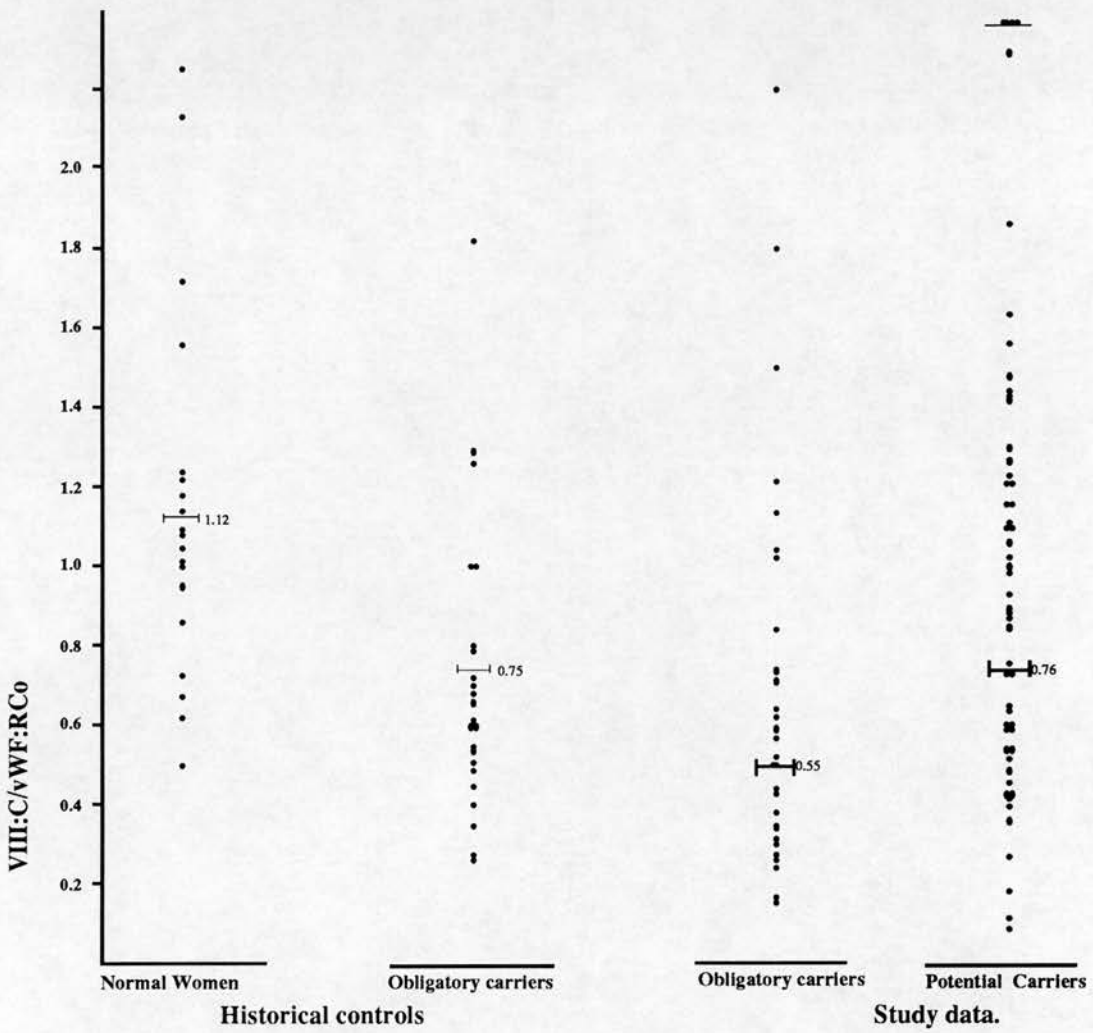


Figure 13. Scattergram showing the distribution of the VIII:C/vWF:RCo ratio in the study obligatory carriers and potential carriers of haemophilia A compared to the historical controls (normal women and obligatory carriers). The mean value for each group is indicated.

3.1.3. Discrimination between Obligate Carriers and Normal Women and the Classification of Potential Carriers of Haemophilia A using the Ratios VIII:C/vWF:Ag and VIII:C/vWF:RCo.

The value of the VIII:C/vWF:Ag and VIII:C/vWF:RCo ratios as discriminants of carriership, are shown in Figures 14-16 where the VIII:C/vWF:Ag ratio is plotted against the VIII:C/vWF:RCo ratio for:

- the historical controls ie. obligate carriers and normal females - Figure 14.
- the study obligate carriers and the historical normal controls - Figure 15.
- the study potential carriers and the historical normal controls - Figure 16.

Lines which completely separate the control groups of normal females and obligatory carriers have been drawn and the ratios at which these lines intercept the axes have been used to assign a phenotype to obligate and potential carriers identified by pedigree analysis within the study. (The limitations of this approach will be discussed in Chapter 4). Only individuals in whom both the ratios could be calculated are shown in these figures. The results of these studies are summarised in Table 21.

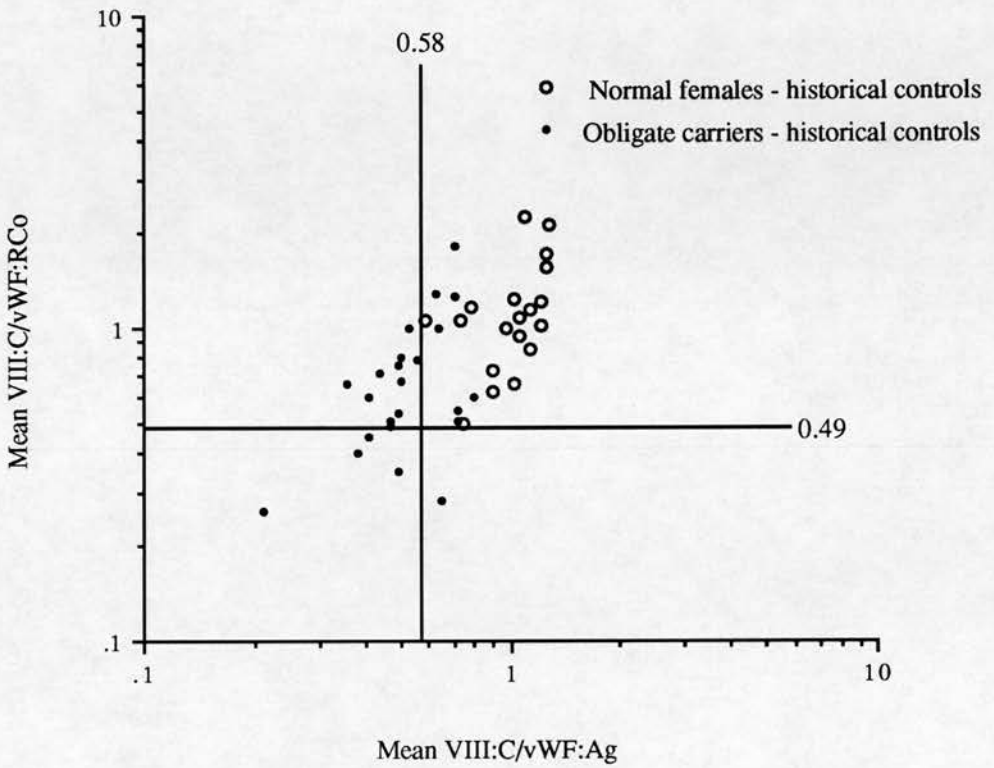


Figure 14. VIII:C/vWF:Ag ratio plotted against VIII:C/vWF:RCo ratio for the historical obligate carriers and historical normal female control group. A line for each ratio has been drawn which separates the normal females from the obligate carriers.

NB. The ratios that separate normal females from obligatory carriers are 0.58 (VIII:C/vWF:Ag) and 0.49 (VIII:C/vWF:RCo). The VIII:C/vWF:Ag ratio of 0.58 misclassifies 8/23 (34.8%) of the obligatory carriers as normal whilst the VIII:C/vWF:RCo ratio of 0.49 misclassifies 18/23 (78.3%) as normal - the VIII:C/vWF:Ag ratio, therefore, appears to be a more sensitive discriminant of carriership.

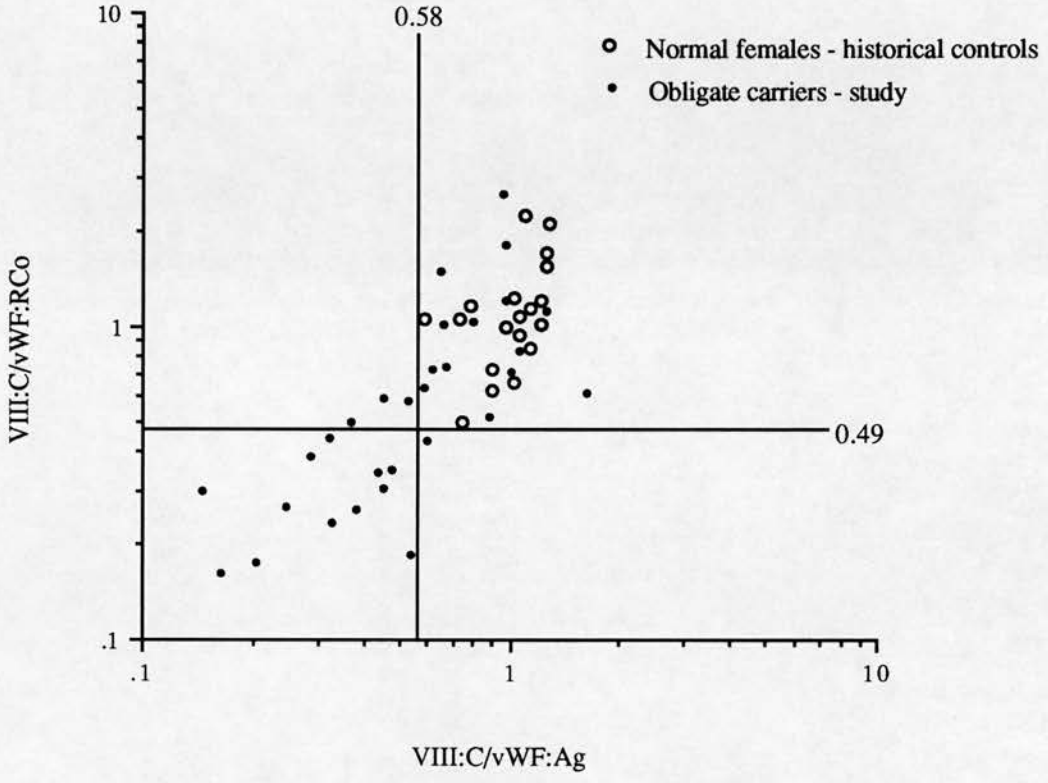


Figure 15. VIII:C/vWF:Ag ratio plotted against VIII:C/vWF:RCo ratio for the historical normal female controls (mean of 3 samples) and the obligate carriers identified within the study (single sample). The lines represent the ratios derived from Figure 14.

NB. The VIII:C/vWF:Ag ratio (<0.58) misclassifies 15/30 of the study obligatory carriers as normal whilst the VIII:C/vWF:RCo (<0.49) ratio misclassifies 17/30 (56.7%) as normal.

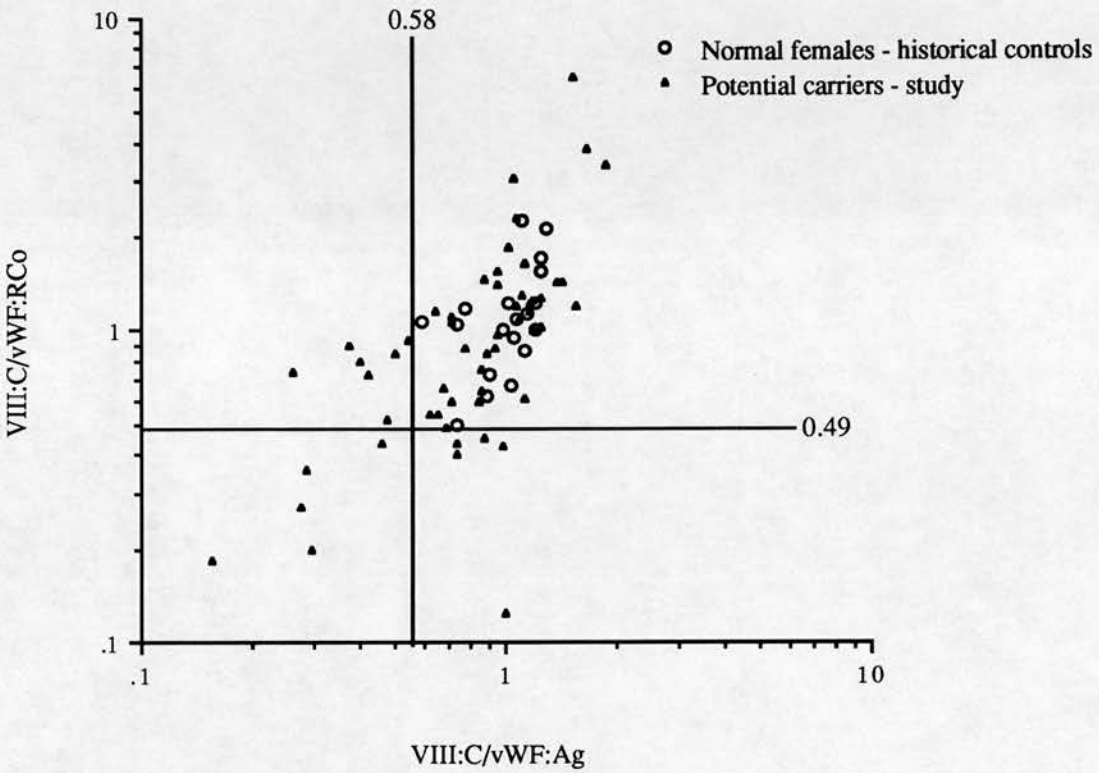


Figure 16. VIII:C/vWF:Ag ratio plotted against VIII:C/vWF:RCo ratio for the normal female controls and the study potential carriers. The lines represent the ratios derived from Figure 14.

NB. Of the potential carriers, 41/53 are identified as normal with the VIII:C/vWF:Ag ratio (<0.58) and 43/53 with the VIII:C/vWF:RCo ratio (<0.49).

Table 21. Summary of data from Figures 14-16.

Subject Group	No. correctly identified by VIII:C/vWF:Ag ratio (<0.58)	No. correctly identified by VIII:C/vWF:RCo ratio (<0.49)
Historical controls.		
[1] Obligate carriers	n=23 15/23 (65.2%)	n=23 5/23 (21.7%)
[1] Normal females	n=31 100%	n=31 100%
Study group.		
[1] Obligate carriers	n=30 15/30 (50%)	n=30 13/30 (43.4%)
[2] Obligate carriers	n=37 21/37 (56.8%)	n=30 13/30 (43.4%)

Classification of potential carriers using the discriminants above.

	Normal	Carrier	Normal	Carrier
[1] Potential carriers	n=53 41/53 (77.4%)	12/53 (22.6)	n=53 43/53 (81.1%)	10/53 (18.9%)
[2] Potential carriers	n=74 54/74 (73%)	20/74 (27%)	n=61 50/61 (82%)	11/61 (18%)

[1] Data for both VIII:C/vWF:Ag and VIII:C/vWF:RCo ratios available.
[2] Data for only one ratio available.
n = number of women for whom ratios could be calculated.

3.1.4. Summary of Data and Interpretation of Results from Figures 14-16.

The data from Figures 14-16 is summarised in Table 21. For the historical controls, data for measurements of both VIII:C/vWF:Ag and VIII:C/vWF:RCo were available but for women within the study, it was not always possible to determine both ratios eg. missing vWF:RCo data. From the results in Figure 14 the ratios were determined that correctly classified all of the normal female controls. Using these ratios to analyse both the obligate carriers (controls and study) and the potential carriers showed:

1. Historical obligate carriers

The VIII:C/vWF:Ag ratio correctly identified 15/23 (65.2%) of the historical obligate carriers, misclassifying 8/23 (34.8%) whilst the VIII:C/vWF:RCo ratio correctly identified only 5/23 (21.7%), misclassifying 18/23 (78.3%). From these findings the VIII:C/vWF:Ag ratio appeared to be a more sensitive discriminant.

2. Obligate Carriers and Potential carriers identified within the Study.

A. Obligate carriers - the VIII:C/vWF:Ag ratio correctly identified 15/30 (50%) of carriers, misclassifying 50%. When the VIII:C/vWF:Ag ratio of 0.58 was used to screen all obligate carriers irrespective of whether the VIII:C/vWF:RCo ratio had been measured, then 21/37 (56.8%) were correctly classified. When the VIII:C/vWF:RCo ratio was used the number of obligate carriers correctly classified fell to 43.4%. These findings support those found for the historical controls in that the VIII:C/vWF:Ag ratio appears to be a more sensitive discriminant of carriership.

B. Potential carriers - using the VIII:C/vWF:Ag ratio of 0.58, predicted that 41/53 (77.4%) of the potential carriers would be normal and 12/53 (22.6%) would be carriers whilst the VIII:C/vWF:RCo ratio predicted that 43/53 (81.1%) would be normal and 10/53 (18.9%) would be carriers. When the VIII:C/vWF:Ag and VIII:C/vWF:RCo ratios were used to screen all potential carriers (irrespective of whether results for both assays were available) the numbers predicted to be carriers were 20/74 (27%) for VIII:C/vWF:Ag and

11/61 (18%) for VIII:C/vWF:RCo.

Phenotypic analysis in families with haemophilia A is invaluable for a number of reasons - it can identify those women with low VIII:C values and, therefore, at risk of bleeding and it can establish which potential carriers are likely to be true carriers but it cannot exclude carriership in potential carriers with normal VIII:C/vWF:Ag and VIII:C/vWF:RCo ratios. However, phenotypic analysis can prove invaluable in the interpretation of the genotypic data and in women in whom genotypic analysis is unhelpful.

3.2. Genotypic Analysis in Haemophilia A.

3.2.1. Intragenic and Extragenic Polymorphisms.

Carrier detection was attempted in 80 'at-risk' females from 44 families with haemophilia A using three intragenic (Bcl I, Xba I, Bgl I) and two extragenic (Bgl II, Taq I) polymorphisms. Forty obligate carriers of haemophilia A were also studied to identify informative RFLP's for use in prenatal diagnosis.

The first probes to be obtained allowed analyses for the Bgl II and Bcl I polymorphisms to be performed and were, therefore, carried out on the majority of families. Families which were informative with only the linked Bgl II marker or non-informative for the Bcl I polymorphism were studied further to determine heterozygosity for the Xba I and/or Bgl I polymorphisms. In cases which were otherwise non-informative, the multiallelic, extragenic Taq I polymorphism was analysed.

Intragenic Polymorphisms.

1. Bcl I polymorphism.

The Bcl I polymorphism, located within intron 18, produces a band of 0.8kb when the site is present and a 1.1kb band when the site is absent after digestion of genomic DNA with Bcl I and probing with p114.12. In initial studies this polymorphism was analysed by Southern blotting but subsequently analyses were performed by enzymatic amplification using the polymerase chain reaction (PCR). Two other polymorphisms are in complete linkage disequilibrium with the Bcl I intron 18 polymorphism - a Hind III polymorphism situated in intron 19[196,233], and an Msp I polymorphism situated in 3' flanking region.[197] As a result of this strong linkage disequilibrium they offer no advantages over the readily analysed Bcl I marker.

Figure 17 shows an autoradiograph from a family in which there was a single affected male and in which the Bcl I polymorphism was informative. From the pedigree (Pedigree No. 43 - Appendix 1), I-1 and II-2 are both potential carriers. Genotypic analysis shows

that the gene in haemophilic II-1 is marked by the 0.8kb fragment of the Bcl I polymorphism; II-2 is homozygous for the 1.1kb fragment, having inherited a 1.1kb fragment from her father (I-2) and the normal 1.1kb fragment from her mother (I-1) and is, therefore, not a carrier.

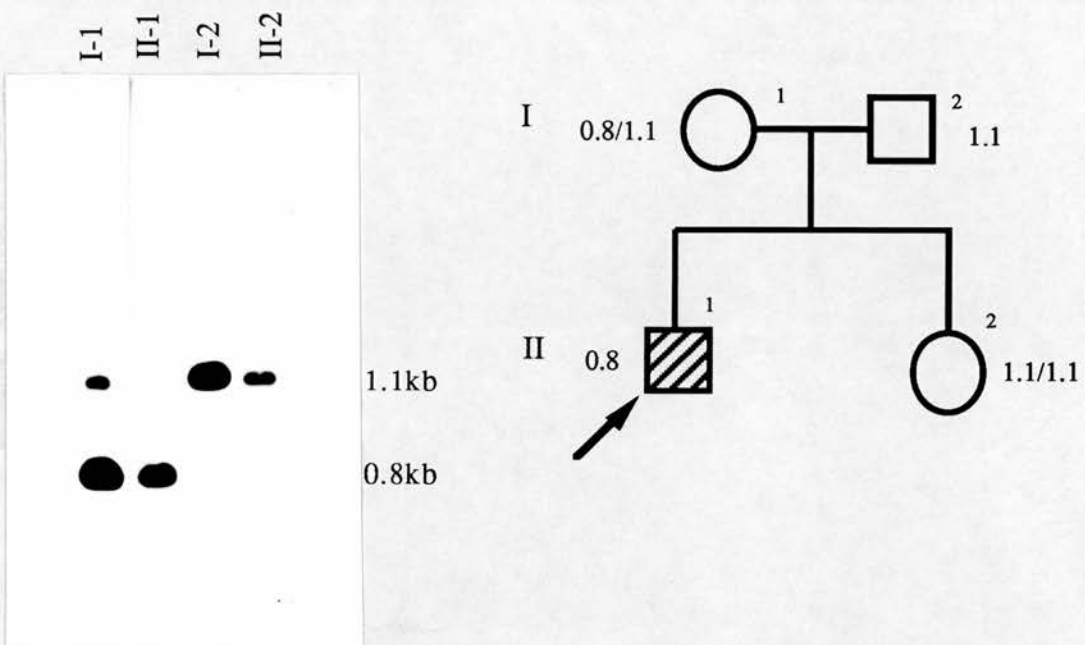


Figure 17. Autoradiograph of leucocyte DNA digested with Bcl I and hybridised with the genomic probe p114.12. The polymorphic bands at 0.8kb and 1.1kb are indicated.

Figure 18 shows part of Pedigree No. 10 (Appendix 1) in which the Bcl I polymorphism has been identified using the Polymerase Chain Reaction. The region containing the Bcl I site was amplified, digested with Bcl I and electrophoresed in a 1.5% agarose gel containing ethidium bromide. Amplification generates a single fragment of 142bp. After digestion with Bcl I if the polymorphic site is present two bands are seen at 99bp and 43bp whilst if the site is absent only a single band of 142bp is visible.

In this pedigree the abnormal gene is associated with the 99/43bp bands (0.8kb by Southern blotting) of the Bcl I polymorphism (III-5). Both I-2 and II-6 are obligatory carriers but only II-6 is heterozygous for the Bcl I polymorphism. II-6's son, III-6 has inherited the normal 142bp fragment and is, therefore, normal. As II-6 is informative for the Bcl I polymorphism this could be used for prenatal diagnosis.

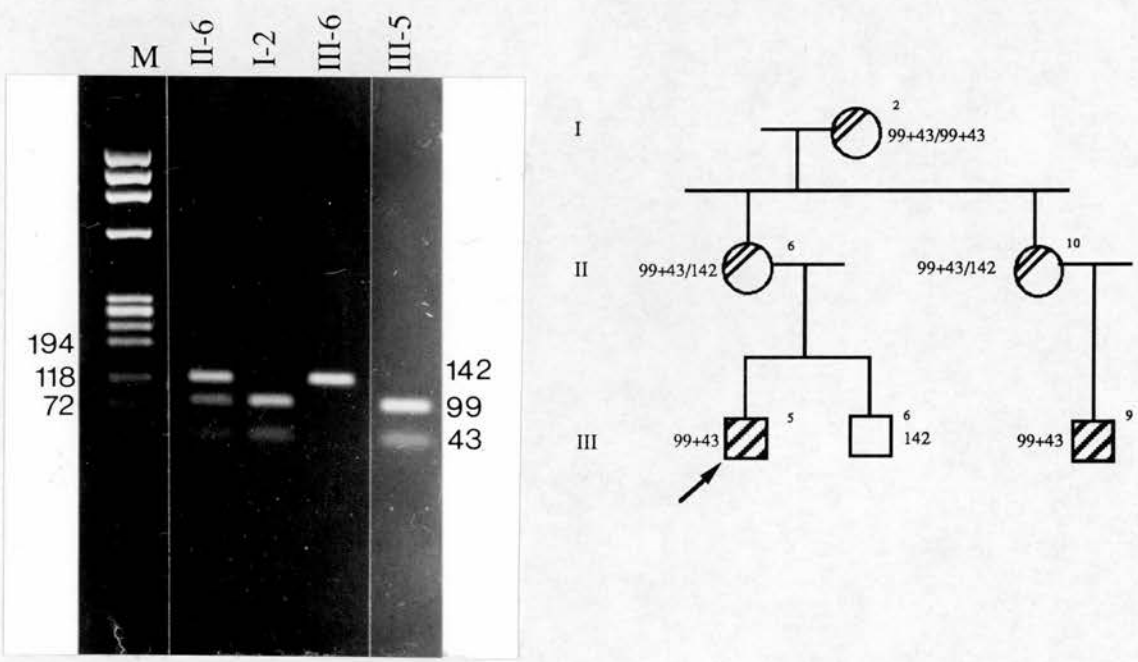


Figure 18. 18µl of a Bcl I amplification was digested with 10units of Bcl I and electrophoresed in a 1.5% agarose gel containing ethidium bromide (0.5µg/ml). A single band of 142bp is seen when the Bcl I site is absent and two fragments at 99bp and 43bp if the site is present. M - ∅X174/Hae III markers.

The Bcl I polymorphism proved useful in a number of situations.

1. No coagulation data available

Intragenic polymorphisms are useful in establishing carrier status in families in which coagulation data is not available on potential carriers and their use is preferred to extragenic polymorphisms as the latter carry a risk of recombination and, therefore, of misdiagnosis.

In Pedigree No. 43 (Appendix 1), although no coagulation data was available it was possible to exclude the 'at-risk' daughter (II-2) as a haemophilia A carrier on the basis of the results obtained by Bcl I analysis even though it was not possible to confirm her mother (I-1) as a carrier.

2. Identifying the origin of the haemophilic mutation.

(See also - Sporadic Haemophilia A).

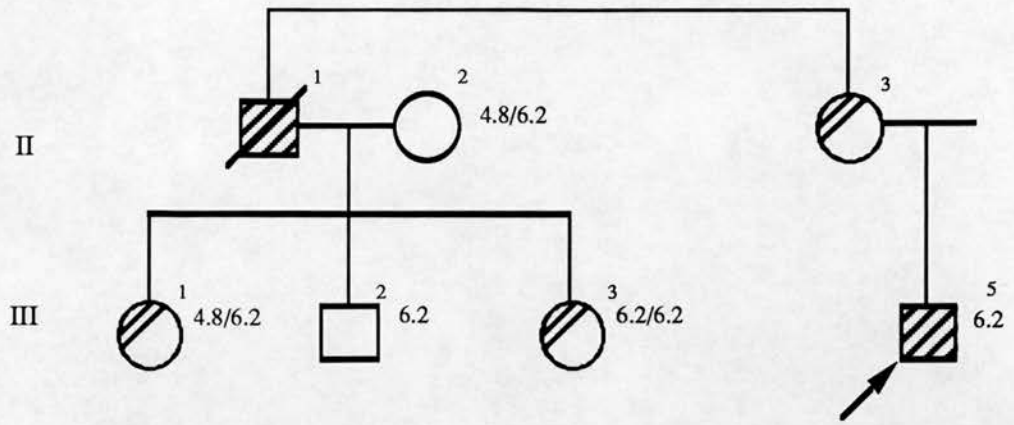
In some families, the presence of an identical haplotype in brothers, one a normal male and the other a haemophiliac, indicated that the mutation was unique to the affected male and excluded other females within the kindred as carriers. For example, in Pedigree No. 20 (Appendix 1), there are 3 potential carriers although all have a normal coagulation phenotype. The haemophilic gene is marked by the 0.8kb (and 2.8kb Bgl II) allele which both III-1 and III-2 have inherited from their mother. This suggests that the mutation is unique to III-1 although it is possible that II-2 could be a germ line mosaic.

2. Xba I polymorphism.

The Xba I polymorphism, situated in intron 22, detects a two allele system after a double digestion of genomic DNA with Xba I and Kpn I and probing with p482.6. A double digestion is required to separate the polymorphic FVIII gene hybridising bands away from a non-FVIII hybridising band.

Figure 19 shows Pedigree No. 35 (Appendix 1) in which the Xba I polymorphism has been analysed by Southern blotting using a EcoR I/Sst I fragment of the genomic clone p482.6 as a probe. This results in two polymorphic fragments of either 6.2kb or 4.8kb and a non-FVIII hybridising band at 6.6kb. In lanes A1-5 5μg of genomic DNA was digested with Xba I and Kpn I whilst in lanes B1-5 10μg of DNA was digested. The polymorphic bands at 4.8kb and 6.2kb are visible as is the non-FVIII hybridising band at 6.6kb. These are more easily seen in panel A than panel B, although the additional polymorphic band at 4.8kb in II-2 is clearer in panel B. The figure illustrates that to resolve the polymorphic 6.2kb fragment from the 6.6kb fragment, the amount of DNA loaded onto the gel is critical and it is, therefore, advisable to perform two digests with varying amounts of DNA.

In Pedigree No. 35, III-1 and III-3 who are both obligate carriers of haemophilia A, sought advice concerning prenatal diagnosis of the disease. The mutant gene is marked by the 6.2kb fragment of the Xba I polymorphism which both III-1 and III-3 must inherit. III-1 is heterozygous for the Xba I polymorphism and can, therefore, be offered antenatal diagnosis but her sister (III-3) is homozygous for this marker and another, informative marker should be sought.



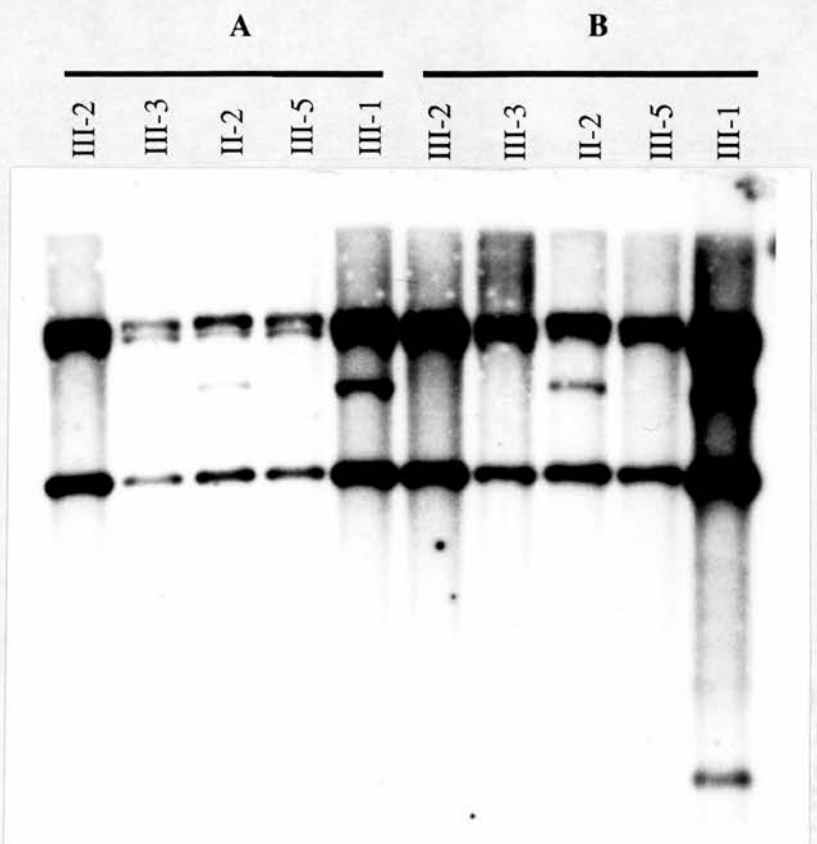


Figure 19. Autoradiograph of leucocyte DNA digested with Xba I/Kpn I and hybridised with an EcoR I/Sst I fragment of the genomic probe p482.6. In panel A, lanes 1-5 contain 5µg of DNA and in panel B 10µg.

With developments in technology it has become possible to analyse the Xba I polymorphisms by means of PCR and this is illustrated in Figure 20. The results are complicated by a non-FVIII sequence which is also amplified and masks the 96bp polymorphic fragment. In some females, depending upon which other family members are available, the Xba I site on the second chromosome may be inferred and in males with only a single X chromosome the Xba I polymorphism can be established. The ease by which families can be screened for heterozygosity at the Xba I polymorphism by PCR is such that all families non-informative for the Bcl I polymorphism are now screened using this technique and those in which Xba I appears to be informative are then reanalysed by Southern blotting.

Figure 20 shows a 12% non-denaturing polyacrylamide gel stained with ethidium bromide showing Pedigree No. 40 (Appendix 1) which was informative for the Xba I polymorphism. The haemophilic gene in this kindred is marked by the 96bp fragment (II-1). Males I-1 and II-1 show only a single band of 96bp and we can infer that they lack the Xba I site ie. 6.2kb by Southern blotting. I-2 and II-2 both show bands at 96bp and 68bp/28bp indicating the presence of an Xba I site. However, it is not clear whether these two females are heterozygotes or homozygotes as the co-amplifying non-FVIII 96bp band masks the other polymorphic site. However, I-2 must be heterozygous as she has a son (II-1) who lacks the Xba I polymorphism and II-2 must also be heterozygous as she must inherit an X-chromosome from her father (I-1) who lacks the Xba I site. This assumes correct paternity.

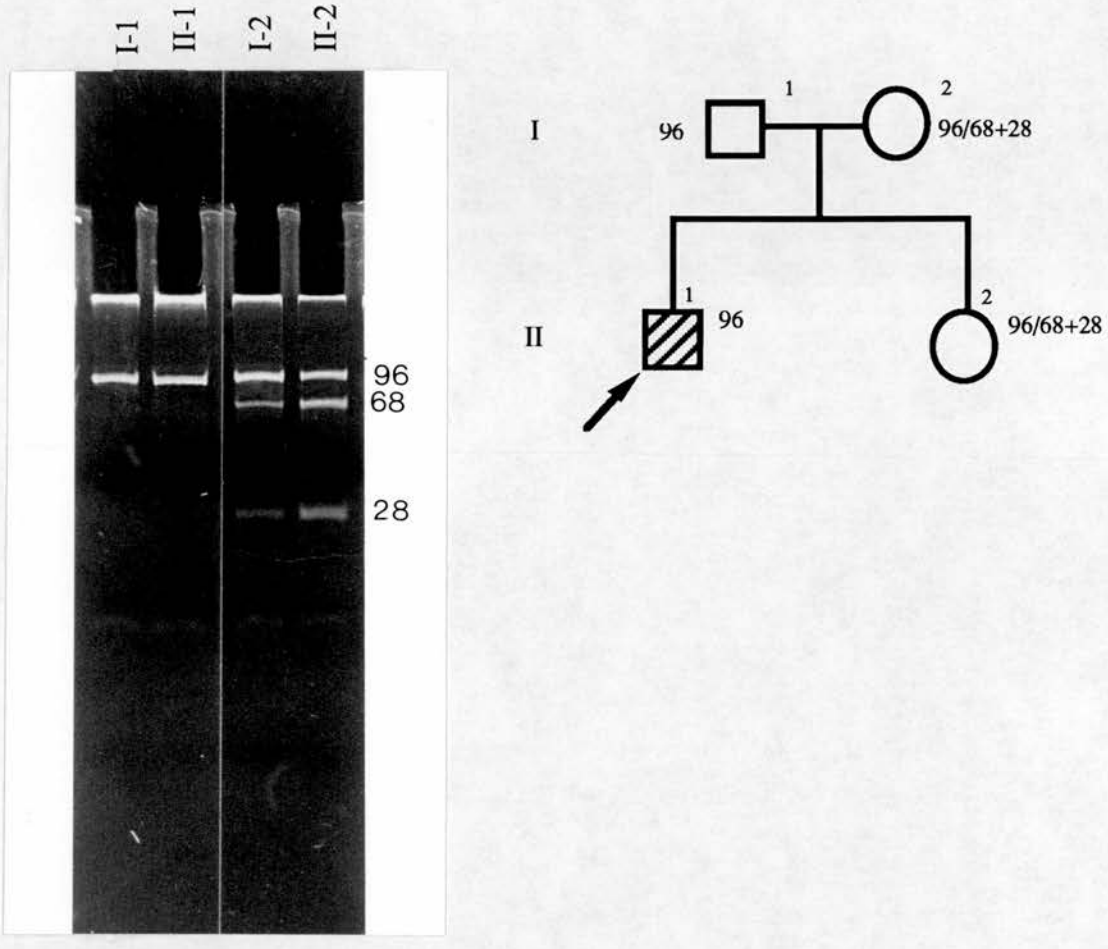


Figure 20. 18µl of a 100µl Xba I amplification was digested with 10units of Xba I and electrophoresed in a 12% non-denaturing polyacrylamide gel in 1X TBE. After electrophoresis the gel was soaked for 20 minutes in 1X TBE containing ethidium bromide (0.5µg/ml). The constant band at 96bp which masks one of the polymorphic fragments is seen as are the two fragments of 68bp and 28bp generated when the Xba I site is present.

3. Bgl I polymorphism.

The Bgl I polymorphism, adjacent to exon 26 detects a two allele system after digestion with Bgl I and probing with the FVIII cDNA probe - Probe C. A band of 20.0kb is seen when the site is absent and 5.0kb when the site is present.

Figure 21 shows an autoradiograph obtained from one of the few families (Pedigree No. 42 - Appendix 1) in which the Bgl I polymorphism was helpful. II-2 is a potential carrier of haemophilia A with an abnormal coagulation phenotype. As her son, (III-1) is phenotypically normal, we can deduce that the haemophilic gene is marked by the 5.0kb fragment. Her daughter III-2 is homozygous for the 5.0kb allele; she must have inherited one allele from her father (II-1) and the other from her mother. Although this does not establish the carrier status of III-2, the finding of abnormal coagulation phenotypes in both mother and daughter supports the suggestion that they are both carriers. Although reported here in the context of haemophilia A, it is possible that this family has a variant of von Willebrand's disease in which there is a mutation in the vWF protein affecting binding of the FVIII molecule. This is discussed in greater detail in Chapter 4.

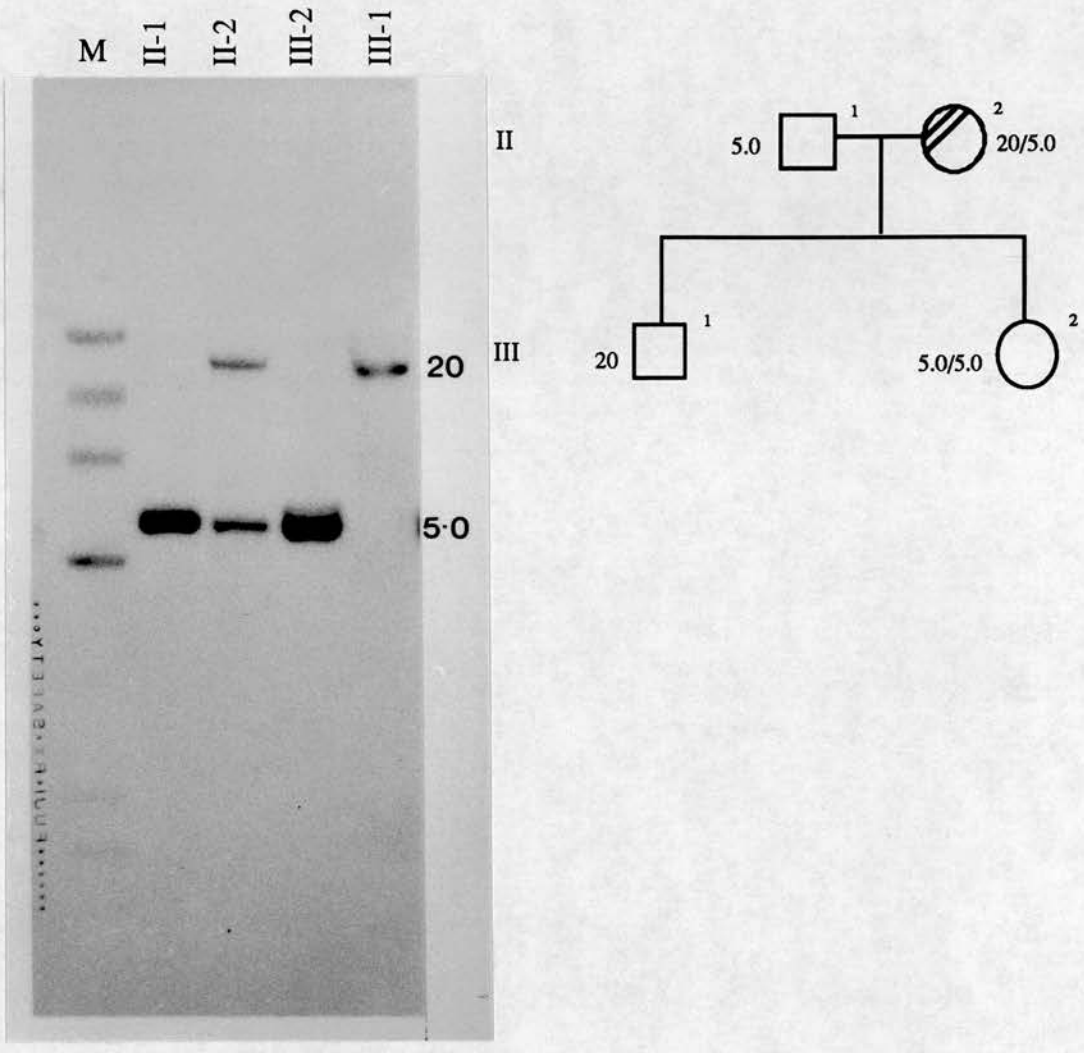


Figure 21. Autoradiograph of leucocyte DNA digested with Bgl I and hybridised with the FVIII cDNA clone - Probe C. The polymorphic fragments at 20.0kb and 5.0kb are clearly visible. M - Lambda DNA/Hind III markers.

3.2.2. Linked or Extragenic Polymorphisms.

Wherever possible informative intragenic polymorphisms were sought to identify carriers. However, a number of women will be non-informative ie. homozygous for all these markers and in these cases, the linked extragenic polymorphisms may be useful. Two extragenic markers were used in the genotypic studies - the Bgl II and Taq I polymorphisms both situated centromeric to the FVIII gene.

1. Bgl II polymorphism.

The probe for this polymorphism became available early in the study and was used to study a number of families before the intragenic markers became available. This extragenic polymorphism detected with the probe DX13 (DXS15) yields fragments of either 2.8kb or 5.8kb and up to 4 constant non-polymorphic bands.

Figure 22 shows Pedigree No. 14 (Appendix 1) in which the intragenic markers were unhelpful but which was informative for the Bgl II polymorphism. The haemophilic gene is marked by the 2.8kb fragment (III-1) for which II-1 is heterozygous. Although this could be used in prenatal diagnosis there would be a 5% chance of error due to possible recombination.

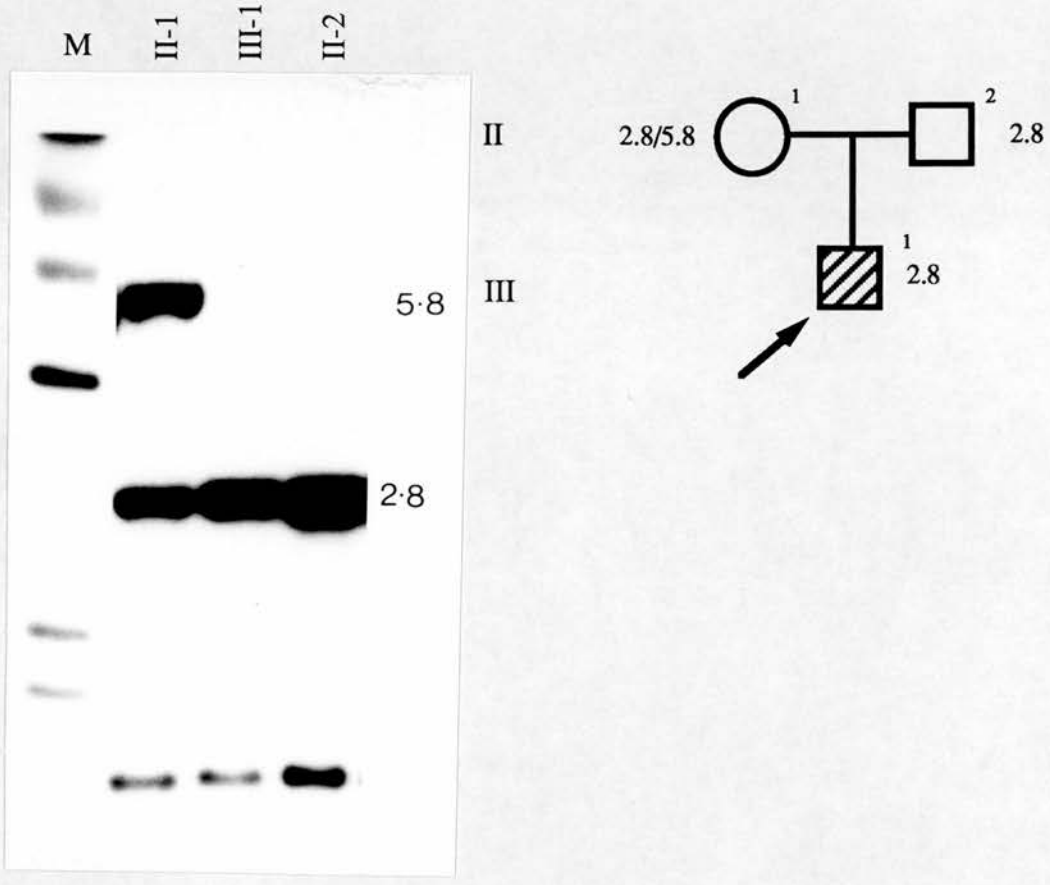


Figure 22. Autoradiograph of leucocyte DNA digested with Bgl II and hybridised with the genomic probe DX13 (DXS15). The polymorphic fragments at 2.8kb and 5.8kb are clearly visible. M - Lambda DNA/Hind III markers.

2. Taq I polymorphism.

The extragenic Taq I marker detected by the probe ST14 (DXS52), yields up to ten polymorphic bands after digestion with Taq I and is of use when the other polymorphisms are non-informative. However, because it is extragenic it is associated with a risk of recombination and, therefore, of misdiagnosis. This polymorphism was analysed in women who were non-informative for the other markers both intra- and extragenic. A major problem with the Taq I polymorphism is the interpretation of blots with supposedly constant fragments migrating together with the polymorphic fragments.

Figure 23 shows Pedigree No. C1 (Appendix 3), a pedigree in which there was no history of haemophilia A or B but in which help was sought in identifying potential carriers in a family with X-linked adrenoleukodystrophy, using the FVIII gene probes. This family is described in more detail at the end of this Chapter. As this disease is X-linked the interpretation of the results is identical to those obtained for haemophilia A and B. Within this family the disease is associated with the 4.5kb allele of the Taq I polymorphism (III-1). III-2 has inherited the same allele from his mother and might be expected to be affected by the disease - a suggestion recently confirmed both clinically and from biochemical analyses. Their mother (II-1) must, therefore, be an obligatory carrier of the disease and this is confirmed by the finding of abnormal long chain fatty acids, in keeping with a carrier state for the disease. She is heterozygous for the Taq I polymorphism having inherited a 4.1kb allele from her father (I-1) and a 4.5kb allele from her mother (I-2). I-2 is a potential carrier of the disease, although no long chain fatty acid studies were available. Her son, II-3 has inherited the 3.9kb allele and is clinically normal but her daughter, II-4 is heterozygous for the Taq I allele having inherited a 4.1kb allele from her father and the 3.9kb allele from her mother. Even though the carrier status of I-2 cannot be definitively established, the haplotype of II-4 is against her being a carrier. The linkage of the ST14 marker to the ALD gene is discussed in greater detail at the end this chapter.

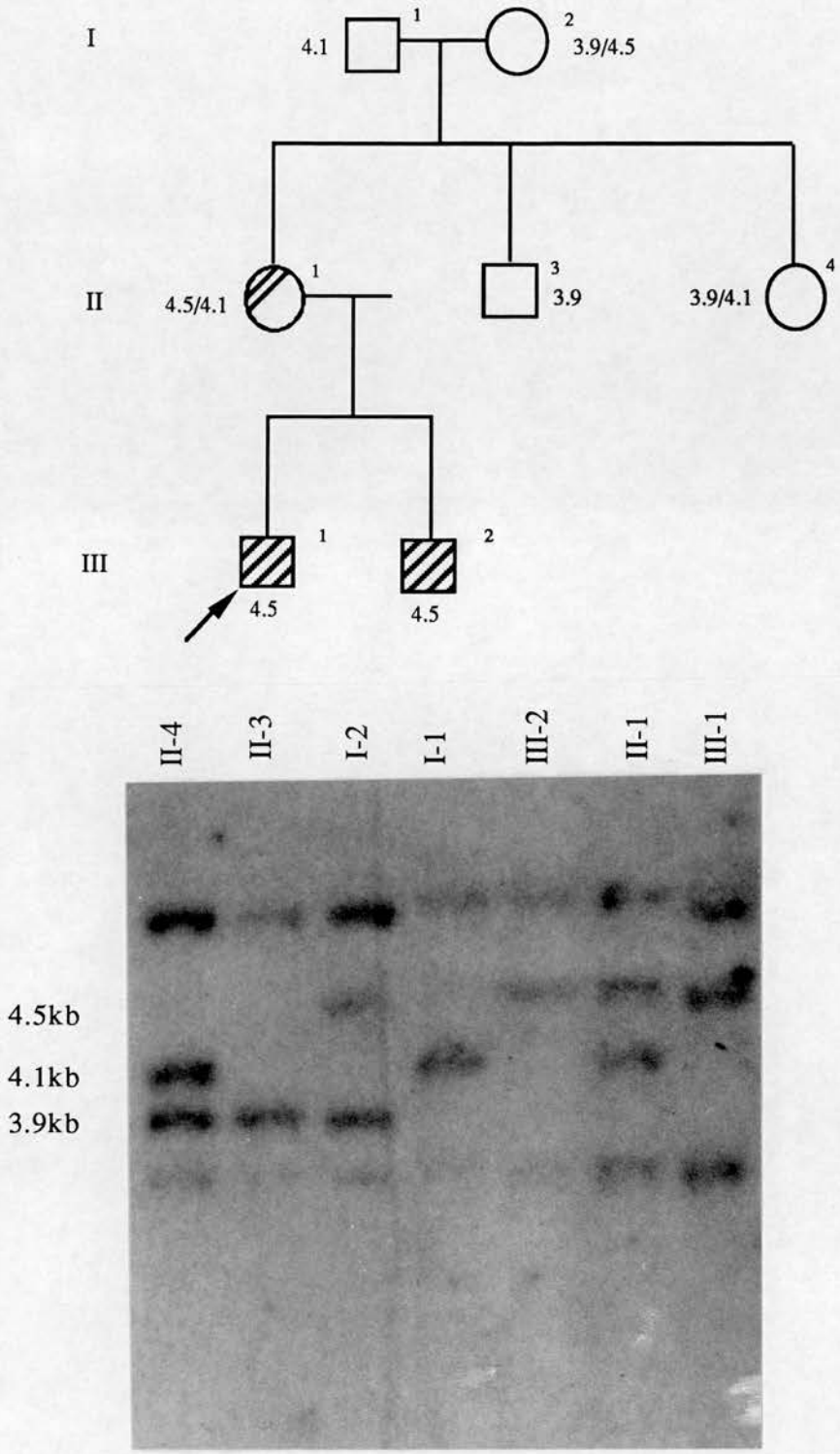


Figure 23. Autoradiograph of leucocyte DNA digested with Taq I and hybridised to the ST14 (DXS52) probe. The polymorphic bands at 4.5kb, 4.1kb and 3.9kb are clearly visible.

3.2.3. Heterozygosity Frequencies for the Intragenic and Extragenic Polymorphisms.

The frequencies of the allelic DNA fragments and the number of heterozygous females for the three intragenic and the two extragenic polymorphisms are summarised in Table 22. This shows the data obtained for all women and does not exclude related individuals. Data for unrelated individuals is shown in Table 23 and gives a more accurate indication of the frequency of heterozygotes.

Not all the available polymorphisms were analysed for each female. As the probes for the Bgl II and Bcl I polymorphisms became available early in the study these analyses were carried out on most women. The Xba I polymorphism was analysed only in women in whom the Bcl I and Bgl I polymorphisms were unhelpful. The Bgl I polymorphism was studied in most women to determine the frequency of the various haplotypes in the local population. Lastly, the Taq I polymorphism was analysed only in women who were non-informative for all other markers.

1. All females (Table 22).

51 of 122 women (41.8%) were heterozygous for the Bcl I polymorphism, 36 of 63 women (57.1%) were heterozygous for the Xba I polymorphism, 10 of 75 women (13.4%) were heterozygous for Bgl I. Of the linked, extragenic markers, 66 of 112 women (58.9%) were heterozygous for the Bgl II polymorphism and all but two women examined for the Taq I polymorphism were heterozygous although only 10 women were analysed.

Table 22. Frequencies of allelic DNA fragments at three intragenic and two extragenic polymorphic sites in or close to the FVIII gene for all women in whom data was available.

RFLP	No. of X chromo-somes analysed	Alleles (kb)	Allele Frequency	Heterozygosity Alleles (kb)	Observed (%)	Predicted (%)
Intragenic polymorphisms						
Bcl I	346	0.8	0.76	0.8/1.1	41.8	36.5
		1.1	0.24			
Xba I	184	4.8	0.58	4.8/6.2	57.1	48.8
		6.2	0.42			
Bgl I	210	5.0	0.90	5.0/20.0	13.4	18
		20.0	0.10			
Extragenic polymorphisms						
Bgl II	325	2.8	0.41	2.5/5.8	58.9	48.4
		5.8	0.59			
Taq I	25	Multiple			80	-

The predicted and observed heterozygosity frequencies are also shown. The predicted heterozygote frequencies are calculated from 2pq x 100 where p and q are the frequencies of the respective alleles eg. for Bcl I the predicted frequency is 2 x 0.759 x 0.241 x 100 = 36.5%.

NB. Heterozygosity frequencies greater than 50% represent sampling errors as the number of heterozygotes cannot exceed 50% in a two allele system.

2. Unrelated females.

Forty-three unrelated women which included obligate carriers, potential carriers and normal females were also studied to determine the heterozygosity frequency in this group (Table 23). The figures are similar to those shown in Table 22.

Table 23. Observed heterozygote frequency for the three intragenic and two extragenic polymorphic sites in or close to the FVIII gene in unrelated women (includes potential carriers, obligate carriers and normal females).

RFLP	No. of X chromo-somes analysed	Alleles (kb)	Allele Frequency	Heterozygosity Alleles (kb)	Observed (%)	Predicted (%)
Intragenic polymorphisms						
Bcl I	44	0.8	0.75	0.8/1.1	36.4	37.5
		1.1	0.25			
Xba I	27	4.8	0.50	4.8/6.2	55.5	50
		6.2	0.50			
Bgl I	31	5.0	0.94	5.0/20.0	13	11.3
		20.0	0.06			
Extragenic polymorphisms						
Bgl II	43	2.8	0.37	2.5/5.8	67	47
		5.8	0.63			
Taq I	4	Multiple	-	-	100	-

NB. Heterozygosity frequencies greater than 50% represent sampling errors as the number of heterozygotes cannot exceed 50% in a 2-allele system

3. Obligatory carriers.

Twenty-four unrelated obligate carriers from the study were also analysed to determine the frequency of heterozygotes in this group (Table 24). The figures are similar to those reported in Tables 22 and 23, although the frequency of heterozygotes is less for the Bgl I polymorphism.

Table 24. Heterozygote frequency for the three intragenic and two extragenic polymorphic sites in or close to the FVIII gene in unrelated obligate carriers of haemophilia A.

RFLP	No. of X chromo-somes analysed	Alleles (kb)	Heterozygosity Alleles (kb)	Heterozygosity Observed (%)
Intragenic polymorphisms				
Bcl I	24	0.8 1.1	0.8/1.1	33
Xba I	19	4.8 6.2	4.8/6.2	58
Bgl I	16	5.0 20.0	5.0/20.0	6.3
Extragenic polymorphisms				
Bgl II	23	2.8 5.8	2.5/5.8	65
Taq I	1	Multiple	-	100

Summary of Tables 22-24.

Tables 22-24 indicate that the intragenic Bcl I and Xba I polymorphisms will be useful in genotypic studies ie. carrier detection and prenatal diagnosis, in both obligate carriers and potential carriers as the observed heterozygosity rate in unrelated females is between 36% (Bcl I) and 55% (Xba I). The Bgl I polymorphism is unlikely to add greatly to the number of women heterozygous for this additional intragenic marker as the frequency of the 20kb allele is low suggesting that the majority of females will be homozygous 5.0/5.0. This is confirmed by the low number of observed heterozygotes (13%). However, the linked Bgl II and the multi-allelic Taq I polymorphisms will be useful in those women in whom the intragenic markers are non-informative. They are unlikely to show linkage disequilibrium with the intragenic markers because of their distance from the FVIII gene and in addition, the numbers of observed heterozygotes in unrelated women is high.

3.2.4. Multiple Heterozygosity.

Some women were heterozygous for more than one polymorphism and this data is shown in Table 25. Ten women were heterozygous for both the Bcl I and Xba I polymorphisms, 9 women for the Bcl I and Bgl I polymorphisms and 26 women for both the Bcl I and Bgl II polymorphisms. Five women were heterozygous for the Bcl I, Xba I and Bgl II polymorphisms and 8 women for the Bcl I, Xba I and Bgl II polymorphisms.

A single woman was informative for the three intragenic polymorphisms and the linked Bgl II marker. No data is available for the Taq I polymorphism as this was only analysed when other markers were non-informative.

Table 25. Number of women heterozygous for more than one polymorphism.

RFLP		Number analysed	Heterozygous Number	Heterozygous %
Bcl I				
	+ Xba I	65	10	15.4
	+ Bgl I	21	9	42.9
	+ Bgl II	46	26	56.5
Bcl I + Xba I				
	+ Bgl I	56	5	8.9
	+ Bgl II	59	8	13.6
Bcl I + Xba I + Bgl I		49	1	2.0

NB. Data shown includes related women.

3.2.5. Frequency of Heterozygotes in Both Unrelated and Related Females, if Non-Informative at the Bcl I and/or Xba I Loci.

The intragenic Bcl I polymorphism was informative in 36.4% of unrelated women (Table 23) and 41.8% of all women (Table 22). Women who were non-informative for this marker were then screened for heterozygosity at the Xba I and Bgl I sites and if these were unhelpful the linked polymorphisms were analysed. The number of women who were informative for both the intragenic and extragenic polymorphisms when either the Bcl I or the Bcl I/Xba I polymorphisms were non-informative are shown in Table 26.

Table 26. Observed frequency of heterozygotes at two intragenic and two extragenic polymorphic sites, when either the Bcl I polymorphism or both the Bcl I and Xba I polymorphisms were non-informative.

	Bcl I non-informative				**Bcl I and Xba I non-informative**			
	All women		**Unrelated**		**All women**		**Unrelated**	
RFLP	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Xba I	23/50	46	12/23	52.1	-	-	-	-
Bgl I	1/54	1.9	1/21	4.8	0/50	-	0/50	-
Bgl II	39/66	59	17/25	68	17/55	31.5	14/25	56
Taq I	-	-	4/4	100	4/5	80	4/4	100

In all women who were non-informative for the Bcl I polymorphism, 46% were informative for the Xba I polymorphism, less than 2% for the Bgl I marker but 59% for the linked Bgl II polymorphism. In all women who were non-informative for both the Bcl I and Xba I polymorphisms, the Bgl I polymorphism was unhelpful as all women were homozygous, however 31.5% of women were heterozygous for the linked Bgl II marker.

The Xba I polymorphism, therefore, proved extremely useful in women who were non-informative for the Bcl I polymorphism. In all women in whom both the Bcl I and Xba I polymorphisms were unhelpful, the Bgl I marker was of little additional benefit. In this latter group, however the linked Bgl II marker was informative in 31.5% of cases. The multi-allelic Taq I marker was analysed in very few families but proved to be of benefit in women homozygous for all other RFLP's.

3.2.6. Distribution of the Bcl I, Xba I and Bgl I Haplotypes in Unrelated Women.

The frequencies of the various haplotypes for the three intragenic polymorphisms in non-related individuals are shown in Tables 27-28 and compared to the distributions expected in the absence of any linkage disequilibrium and to previously published data.

Table 27. Distribution of the Bcl I/Xba I haplotypes in unrelated females.

Bcl I/Xba I haplotype	No.	Observed (%)	[1]Predicted (%)	[2]Wion et al[195] No.	(%)	[2]Moodie et al[334] No.	(%)	Cumulative summary No.	(%)
0.8/4.8	25	62.5	37.5	45	61	37	44	107	54
0.8/6.2	9	22.5	37.5	15	20	23	27	47	24
1.1/4.8	0	0	12.5	0	0	8	10	8	4
1.1/6.2	6	15	12.5	14	19	16	19	36	18
Total	**40**	**100**	**100**	**74**	**100**	**84**	**100**	**198**	**100**

[1]Predicted heterozygosity frequencies calculated from the allelic frequencies detailed in Table 23.

[2]Observed haplotype frequencies

Table 28. Distribution of the Bgl I RFLP alleles with the Xba I/Bcl I haplotypes in unrelated females.

Bcl I/Xba I haplotype	Bgl I allele	Observed		[1]Predicted	[2]Moodie et al[334]		Cumulative summary	
		No.	(%)	(%)	No.	(%)	No.	(%)
0.8/4.8	**5.0**	22	58	35.25	33	43.4	55	48.2
	20	2	5.3	2.25	1	1.3	3	2.6
0.8/6.2	**5.0**	8	21	35.25	20	26.4	28	24.6
	20	1	2.6	2.25	1	1.3	2	1.8
1.1/4.8	**5.0**	0	0	11.75	4	5.3	4	3.5
	20	0	0	0.750	1	1.3	1	0.9
1.1/6.2	**5.0**	3	7.9	11.75	11	14.4	14	12.3
	20	2	5.2	0.75	5	6.6	7	6.1
Total		**38**	**100**	**100**	**76**	**100**	**114**	**100**

[1]Predicted frequency of the various haplotypes in the absence of any linkage disequilibrium, calculated from the haplotype and allelic frequencies detailed in Tables 27 and 23 respectively.

[2]Observed haplotype frequencies

The observed haplotype frequencies reported in Table 27 are similar to those published by Wion et al[195] and indicate linkage disequilibrium - a not unexpected finding as the two polymorphic sites lie within 18kb of each other. The result for the 1.1/4.8kb haplotype suggests a degree of linkage disequilibrium not found by Moodie.[334]

In Table 28, the frequencies of the Bgl I alleles with the various Bcl I/Xba I haplotypes are shown both for the observed haplotypes and those predicted to occur in the absence of any linkage disequilibrium. These results are, in general, similar to those reported by Moodie[334] although no examples of the 1.1/4.8/5.0kb or 1.1/4.8/20kb haplotypes were found. In addition, the 1.1/4.8kb and 1.1/6.2kb haplotypes and either of the Bgl I alleles occur less frequently than predicted suggesting linkage disequilibrium. Overall, the 0.8/4.8/5.0kb and the 0.8/6.2/5.0kb haplotypes accounted for 79% of cases.

These findings suggest strong linkage disequilibrium between the Bcl I, Xba I and the Bgl I polymorphisms and that the value of the Bgl I RFLP in non-informative Bcl I/Xba I haplotypes will be limited - a finding already shown in Table 26. All women identified within the study as informative for Bgl I were also heterozygous for Bcl I. The value of the Bgl I polymorphism in the population studied is, therefore, limited.

3.2.7. Linkage of the FVIII Locus to Other Markers.

The major problem with the use of linked, extragenic markers in haemophilia A is the possibility of a recombination occurring between the FVIII gene and the site of the polymorphism. The chances of such an event occurring are related to the distance between the marker used to track the gene and the gene in question and increases as the distance between the two increases. In this study the linkage of the FVIII locus to the two extragenic polymorphic loci was studied wherever possible. It should be noted that paternity testing was not undertaken in any of these studies.

1. Linkage of the DX13 (DXS15) Locus to the Bcl I Locus.

There were 40 meioses in which it was possible to establish whether a recombination had occurred between the Bcl I and DX13 loci. Within this study, three families (Pedigree Nos. 16, 28 and 32: Appendix 1) were identified in which a recombination event was found to have occurred. This gives an overall recombination rate of 7.5%.

In Pedigree No. 16 the recombination can be shown to have occurred between the extragenic Bgl II site and the Bcl I/Bgl I sites as both intragenic polymorphisms are in agreement.

2. Linkage of the DX13 (DXS15) Locus to the Xba I Locus.

No recombination events between the Xba I and DX13 loci were found in 12 meioses in

which it was possible to study both markers.

3. Linkage of the ST14 (DXS52) Locus to the Bcl I and/or Xba I Loci.

In Pedigree No. 39 (Appendix 1) a possible recombination between the ST14 and Bcl I loci was suspected but the genotypic data available was insufficient to allow a definite decision to be made. The possibility of non-paternity to explain the genotypic findings in this family is discussed with the Pedigree in Appendix 1.

The data available on ST14/Bcl I recombinations is limited as the ST14 locus was only examined when the other polymorphisms were non-informative. Only six meioses were available in which it was possible to study linkage between the ST14 and Xba I loci but no recombinations were observed.

4. Linkage of the DX13 (DXS15) Locus to the ST14 Locus (DXS52).

A single family was found in which a recombination event was thought to have taken place between the DX13 and the ST14 loci - Pedigree No. C1 (Appendix 3). Carrier detection was attempted in this family with X-linked adrenoleukodystrophy (but no history of haemophilia A or B) using both intragenic and extragenic FVIII gene probes.

The pedigree shows a recombination to have taken place in II-4. Her mother (I-2) has haplotypes [5.8/4.5] and [2.8/3.9] whilst her father (I-1) has the haplotype [2.8/4.1]. II-4 has a [2.8/4.1] haplotype from her father and hence a recombinant [5.8/3.9] chromosome from her mother.

The ST14(DXS15) and DX13(DXS52) loci are located close to each other on the X-chromosome; the chances of recombination would appear to be small and it is essential, therefore, to exclude non-paternity.

3.3. Genotypic Analysis in Potential Carriers of Haemophilia A.

Pedigree analysis identified 80 potential carriers in whom carrier detection could be attempted by RFLP analysis. 32 of these potential carriers were from families in which there was a history of haemophilia A affecting more than a single generation or in which the birth of two affected children identified their mother as an obligate carrier. The remaining potential carriers (48) were from families in which there was only a single isolated affected male. This latter group will be discussed in more detail in a separate section on sporadic disease.

Of the 80 potential carriers (Table 29), 47 had a normal coagulation phenotype, 27 were abnormal suggesting carriership and in 6 no coagulation data was available although genotypic analysis was still possible. From phenotypic studies the potential carriers could, therefore, be assigned to one of three groups; normal phenotype, abnormal phenotype and no phenotype data available. Within each of these groups the benefits from genotypic analysis was examined.

1. Normal phenotype: of the 47 potential carriers with a normal coagulation phenotype genotypic data allowed 25 to be classified as normal and only 1 to be re-classified as a carrier. Of the 25 genotypically normal women, 14 were from families in which there was a history of haemophilia and 11 were from families in which there was no previous history. In the remaining 21 phenotypically normal women their carrier status could not be established from the genotypic data either because insufficient family members were available or individuals were homozygous for the polymorphisms analysed or diagnosis by exclusion of the potentially abnormal haplotype was not possible.

2. Abnormal phenotype: of the 27 potential carriers in whom the coagulation phenotype was abnormal, 4/27 were shown to be carriers on the basis of their genotype and all of these were from families in which there was a history of haemophilia. In 4 cases

genotypic analysis allowed exclusion of a potential carrier with an abnormal phenotype. In the remaining 19 women their carrier status could not be confirmed by genotypic analysis although their coagulation phenotype was abnormal and it is likely that this group contains some 'true' carriers.

3. No phenotype data: in 6 women, no coagulation data was available to establish their risk of carriership phenotypically. However, genotypic analysis allowed 2 women to be classified as carriers, 2 to be excluded as carriers but in the remaining 2 women carriership could not be established.

From the data summarised in Table 29 it proved impossible to establish the carrier status in 42/80 (52.5%) of the potential carriers either because key family members were unavailable, or the polymorphic markers studied were unhelpful or diagnosis by exclusion of the potentially abnormal haplotype was not possible.. In these individuals, where the origin of the haemophilic mutation is difficult to establish, exclusion of the mutant haplotype is the only method of carrier detection by RFLP analysis. Therefore, in families in which RFLP analysis is unhelpful and ideally in all families seeking carrier detection, identification of the kindred-specific mutation should be attempted although this is not always possible.

Table 29. Potential carriers classified by their coagulation phenotype and the results following genotypic analysis.

		Carrier status following genotypic analysis		
Phenotype	**Number**	**Carrier**	**Normal**	**Non-informative/Other**
Normal	47	1/47	25/47	21/47
Family History:	18	Family History: 1	Family History: 14	Family History: 3
No Family History:	29		No Family History: 11	No Family History: 18
Normal	27	4/27	4/27	1927
Family History:	10	Family History: 4	Family History: 2	Family History: 4
No Family History:	17		No Family History: 2	No Family History: 15
Normal	6	2/6	2/6	2/6
Family History:	4	Family History: 2	Family History: 1	Family History: 1
No Family History:	2		No Family History: 1	No Family History: 1
Total				
Normal	**80**	**7/80**	**31/80**	**42/80**
Family History:	**32**	**Family History: 7**	**Family History: 17**	**Family History: 8**
No Family History:	**48**		**No Family History: 14**	**No Family History: 34**

3.3.1. Sporadic Haemophilia A.

Estimates of the incidence of sporadic haemophilia A vary widely. In this study there were 18 families in whom no previous history of haemophilia could be elicited. Prior to the advent of recombinant DNA technology, pedigree information and phenotypic analysis were the only methods available to determine the probability of carriership.

In cases in which no family history is available, it is usually only possible to detect non-carrier status using RFLP analysis ie. haplotype exclusion, as determining carrier status depends upon knowing in which generation the new mutation arose.

3.3.2. Phenotypic Analysis in Sporadic Haemophilia A.

The eighteen families comprised a total of 86 individuals of whom 38 were males and 48 female. Of the males 10 had severe disease (VIII:C <0.01iu/dl), 6 had moderately severe disease, 1 was mild (VIII:C=0.09iu/dl) and the remaining 21 were normal. Of the females coagulation data was not available on 2 women. Without genotypic analysis all the women within a kindred with spontaneous haemophilia A must be considered as potential carriers.

The mean VIII:C for the females was 0.81iu/dl, the mean vWF:Ag was 1.08iu/dl and the mean vWF:RCo 1.02iu/dl. Using the VIII:C/vWF:Ag ratio (<0.58) 16/46 (34.8%) of these potential carriers were classified as carriers and 18/46 (39%) using the VIII:C/vWF:RCo ratio (<0.49). From these results it was predicted that almost 35% of the potential carriers from families with sporadic haemophilia A would be carriers.

3.3.3. Genotypic Analysis in Sporadic Haemophilia A.

The results of genotypic analysis in families with isolated affected male are shown in Table 29. Using a combination of intragenic and extragenic polymorphisms, carriership could be excluded in 14/48 (29.2%) of the potential carriers. It was impossible to establish the carrier status of a further 34 women, either because key family members were unavailable, the polymorphisms analysed were unhelpful or because the haemophilic haplotype could not be excluded. It is possible that by studying other family members and/or increasing the numbers of polymorphisms analysed, the number of potential carriers in whom carriership could be accurately established could be increased.

3.3.4. Combined Coagulation and RFLP Analysis in Potential Carriers of Haemophilia A.

Combined phenotypic and genotypic analysis identified 10 potential carriers from families with an isolated affected male, as carriers. In each case they had a carrier phenotype and had inherited the potentially abnormal haemophilic haplotype. Overall, therefore, 24/48 (50%) of the potential carriers could be offered accurate genetic counselling. (5 potential carriers from families with a history of haemophilia were also identified as carriers after combined phenotypic/genotypic analysis).

3.3.5. Origin of the Mutant Gene.

In 8 of the 22 families in which haemophilia A affected only a single generation, RFLP analysis allowed a prediction to be made as to the origin of the mutant gene. In the remaining families, either the pedigree data was too limited key family members were missing or the genotypic data was unhelpful.

Of these 8 families (Pedigree Nos. 5, 9, 11, 12, 20, 25, 31 and 33), in 2 (Pedigree Nos. 5 and 20) the mutation was thought to be unique to the haemophiliac by the finding of a

normal brother with an identical haplotype and, therefore, excluded carriership in any females within those kindred. In a further 3 families (Pedigree Nos. 9, 11 and 12) the mutation could be localised to the maternal grandfather and in each case his daughter ie. the haemophiliac's mother had an abnormal coagulation phenotype suggestive of carriership. In a further 2 families (Pedigree Nos. 25 and 33) although genotypic data was not available on the maternal grandfather, sufficient pedigree data was available to establish that the mutation was likely to have originated with him. In the remaining family (Pedigree No. 31) the mutation was thought to be present in the maternal grandmother and this was supported by an abnormal coagulation phenotype.

These results emphasise the importance of extended pedigree data in families in which the disease appears to be spontaneous, and in particular genotypic analysis of parents, grand-parents and any siblings. It is possible that by extending the family pedigree data, the number of families in which this type of analysis could be performed could be increased.

3.4. Antenatal Diagnosis.

In addition to establishing the carrier status of potential carriers, the availability of antenatal diagnosis for both obligate, established and potential carriers was studied using informative polymorphisms. For the purposes of antenatal diagnosis women could be divided into three groups:

1. Antenatal diagnosis possible using intragenic polymorphisms.

This is possible for obligatory carriers or potential carriers identified by genotypic analysis as carriers and involves the use of informative, intragenic polymorphisms and fetal sexing on chorionic villus samples.

2. Antenatal diagnosis possible using linked, extragenic polymorphisms.

This group of women includes both obligate carriers and genotypically proven carriers who are non-informative for the intragenic polymorphisms and in addition, includes a

group of women whose carrier status could not be accurately established. For these women chorionic villus sampling (CVS) permits early fetal sexing and genotypic analysis of male embryos might exclude a fetus as a haemophiliac. However, because of the risks of error due to recombination using linked RFLP's fetal blood sampling is desirable to confirm the results of genotypic analysis.

3. Antenatal diagnosis possible by fetal blood sampling only.

In some women, non-informative for the polymorphisms analysed, antenatal diagnosis could only be offered by fetal blood sampling after early fetal sexing. Early fetal sexing by CVS or amniocentesis would eliminate the need for fetal blood sampling in approximately 50% of pregnancies.

The availability of antenatal diagnosis and its method for 89 'at-risk' women identified in the study has been considered. Of these women, 40 were obligatory carriers and the remainder were potential carriers in whom genotypic analysis had not confidently excluded carriership.

Tables 34-36 (at the end of this Chapter) summarise the pedigree, phenotypic and genotypic data for each family and shows the availability of prenatal diagnosis to 'at-risk' women. Prenatal diagnosis using genotypic analysis exclusively was available to 33/89 (37%) women; using linked, extragenic markers and fetal blood sampling to a further 39 women (43.8%) and finally to 17 women (19.2%) using fetal blood sampling alone.

3.5. Section 2.
The Results of Carrier Detection Studies in Haemophilia B (Christmas Disease).

Introduction.

With developments in molecular biology and in particular the Polymerase Chain Reaction it is now possible to identify and characterise kindred specific defects in haemophilia B, thereby increasing both the accuracy and availability of carrier detection. However, it is only relatively recently that such techniques have become widely available and this study has, therefore, concentrated on the use of RFLP analysis in carrier detection.

Only 5 families with haemophilia B consented to be studied. A history of haemophilia affecting more than a single generation was obtained in 2 families whilst in a further 2 the disease appeared to have arisen spontaneously. Of these 4 families (Table 30), 22 individuals were analysed, comprising 11 males and 11 females. There were 5 haemophiliacs, all of whom had IX:C values above 0.01iu/dl. Of the 11 females studied, from the pedigree data 3 could be classified as obligate carriers and 8 as potential carriers. A fifth family (Pedigree No. B5) in which a single female was identified with low levels of both IX:C and IX:Ag but no family history, was studied in some detail and the findings are presented at the end of this Chapter. No female could be excluded as a carrier by pedigree analysis alone.

Because of the small number of families available for study, the data on obligatory carriers is limited. Although fifteen normal females were included as a control group for the measurement of IX:C, there was no control group of obligatory carriers and no control data was available for the measurements of IX:Ag.

Table 37 at the end of this Chapter, summarises all the data on the families with haemophilia B whilst the pedigree, phenotypic and genotypic data is contained in Appendix 2.

Table 30. Numerical summary on 22 individuals from 4 families with haemophilia B (Christmas disease).

Number of males studied.

Normal:	6	
Haemophiliac:	5	1 IX:C >0.01<0.05iu/d
		4 IX:C >0.05iu/dl
Total:	**11**	

Number of females studied.

Normal:	0
Potential carriers:	8
Obligate carriers:	3
Total:	**11**

NB. A single female identified as having Christmas disease (Pedigree No. B5) is not included in the above table.

3.5. Analysis and Interpretation of the Phenotypic Data from the Haemophilia B Kindred.

3.5.1. IX:C and IX:Ag in Obligatory and Potential Carriers of Haemophilia B.

IX:C and IX:Ag levels together with their ranges, and means were available for the 3 obligate carriers and 4 of 8 potential carriers (4/8 IX:C; 2/8 IX:Ag) identified within the study and are shown in Table 31 below and Figure 24. Data on 15 normal females is included for comparison.

The mean IX:C results for the 3 obligate carriers was 0.52iu/dl and although similar to that of the potential carriers (0.50iu/dl) is lower than the mean of the normal females (1.10iu/dl). The IX:Ag values were comparable to the IX:C assays in all individuals in whom both measurements were available and no CRM^+ families were detected (Figure 24).

Table 31. IX:C and IX:Ag levels in obligate and potential carriers from the 5 families with haemophilia B.

	IX:C (iu/dl)			IX:Ag (u/dl)		
Category	**Number**	**Mean**	**Range**	**Number**	**Mean**	**Range**
Obligate Carriers	3	0.52	0.16-1.20	3	0.48	0.12-0.88
Potential Carriers	4	0.50	0.32-0.80	2	0.35	0.30/0.40
Normal Females	15	1.10	0.64-1.61	-	-	-

NB. The IX:C data on 15 normal females is included for comparison.

Figure 24A

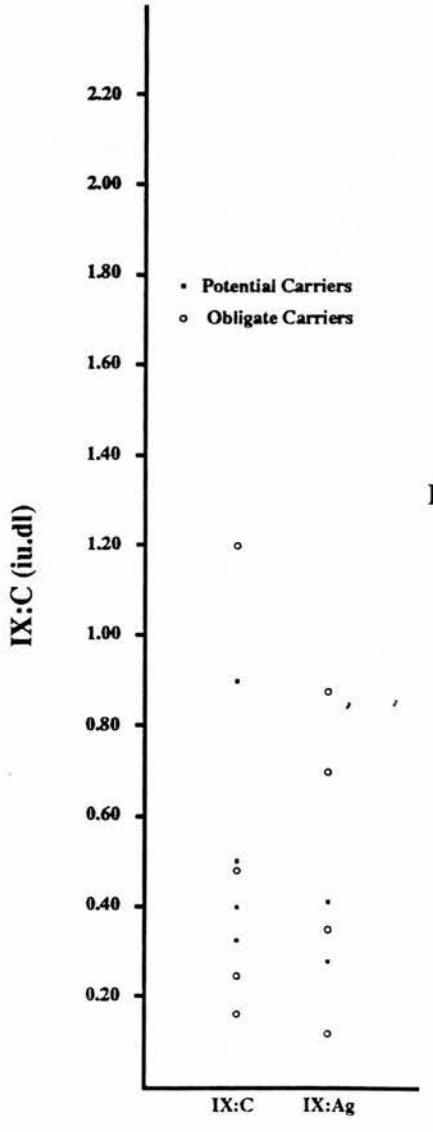


Figure 24B

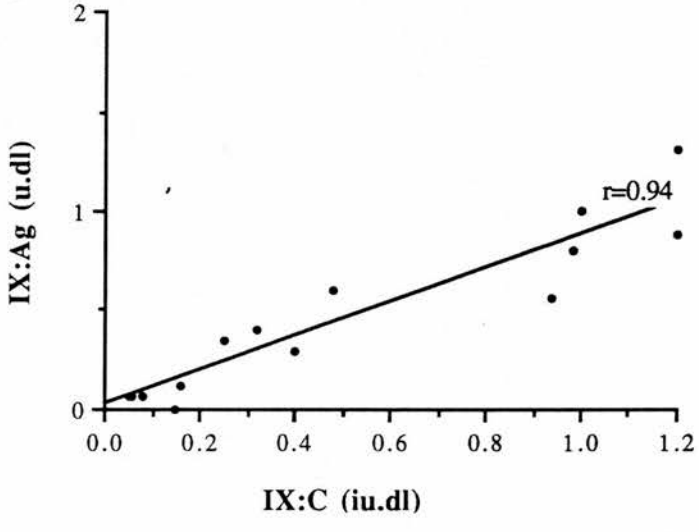


Figure 24. A) The distribution of IX:C and IX:Ag results in potential and obligate carriers of haemophilia B identified within the study. B) Correlation between IX:C and IX:Ag assays in 15 individuals in whom both results were measured.

(Data shown includes the propositus from Pedigree No. B5.)

3.5.2. Assessment of the Haemostatic Risk in Carriers of Haemophilia B using IX:C Assays.

Three of the seven women in whom coagulation data was available had IX:C levels less than 0.40iu/dl and were, therefore, at risk of haemorrhage after trauma or surgery including antenatal diagnosis.

3.5.3. Discrimination between Obligate Carriers of Haemophilia B and Normal Women using IX:C Assays.

Table 33 and Figure 24 show that although the mean IX:C for the obligatory carriers is lower than that of the control group, there is a considerable overlap of values. Limited phenotypic data is available but 2 of 3 obligate carriers and 3 of 4 potential carriers have a IX:C assay below the lowest IX:C value obtained in the control group of 15 normal women (0.64iu/dl) suggesting carriership. Insufficient data was available to allow classification by IX:Ag alone or in combination with measurements of IX:C.

3.6. Genotypic Analysis in Haemophilia B.

3.6.1. Genotypic Analysis in Haemophilia B.

DNA was analysed from 23 individuals from 5 families with haemophilia B and carrier detection was attempted in 8 'at-risk' females. Three intragenic polymorphisms (Taq I, Xmn I and Dde I) were used for gene tracking studies although only 1 family (Pedigree No B2) was analysed for the Dde I polymorphism. No linked polymorphisms were analysed in this study as all families were informative for the common intragenic polymorphisms.

3.6.2. Intragenic polymorphisms.

1. Taq I/Xmn I polymorphisms.

The Taq I polymorphism situated 3' of exon D in the FIX gene, comprises a two allele system. Southern blotting and probing with either a genomic probe (Probe VIII) or the FIX cDNA, produces a 1.8kb band when the polymorphic site is absent and a 1.3kb fragment when present.

The Xmn I polymorphism situated 3' of exon C and close to the Taq I polymorphic site, results in bands of 11.5kb when the site is absent and 6.5kb when the site is present. This polymorphism is detected using the same probes as those used with the Taq I RFLP. Because of the close proximity of the two polymorphic sites, they show strong linkage disequilibrium and as a result each rarely gives any additional information over the other but as both can be detected using the same probe, both were analysed.

Figure 25 shows a Pedigree No. B3 (Appendix 2) informative for both the Taq I and Xmn I polymorphisms. The mutant gene is marked by the 6.5kb and 1.3kb fragments of the Xmn I and Taq I polymorphisms (IV-1 and IV-3) respectively. Females III-2 and II-2 are both obligate carriers. III-2 has two sons both of whom are affected and a single son, IV-2 who has inherited the non-haemophilic 1.8/11.5kb haplotype and is, therefore, normal. Either RFLP could be used for prenatal diagnosis in II-2 or III-2.

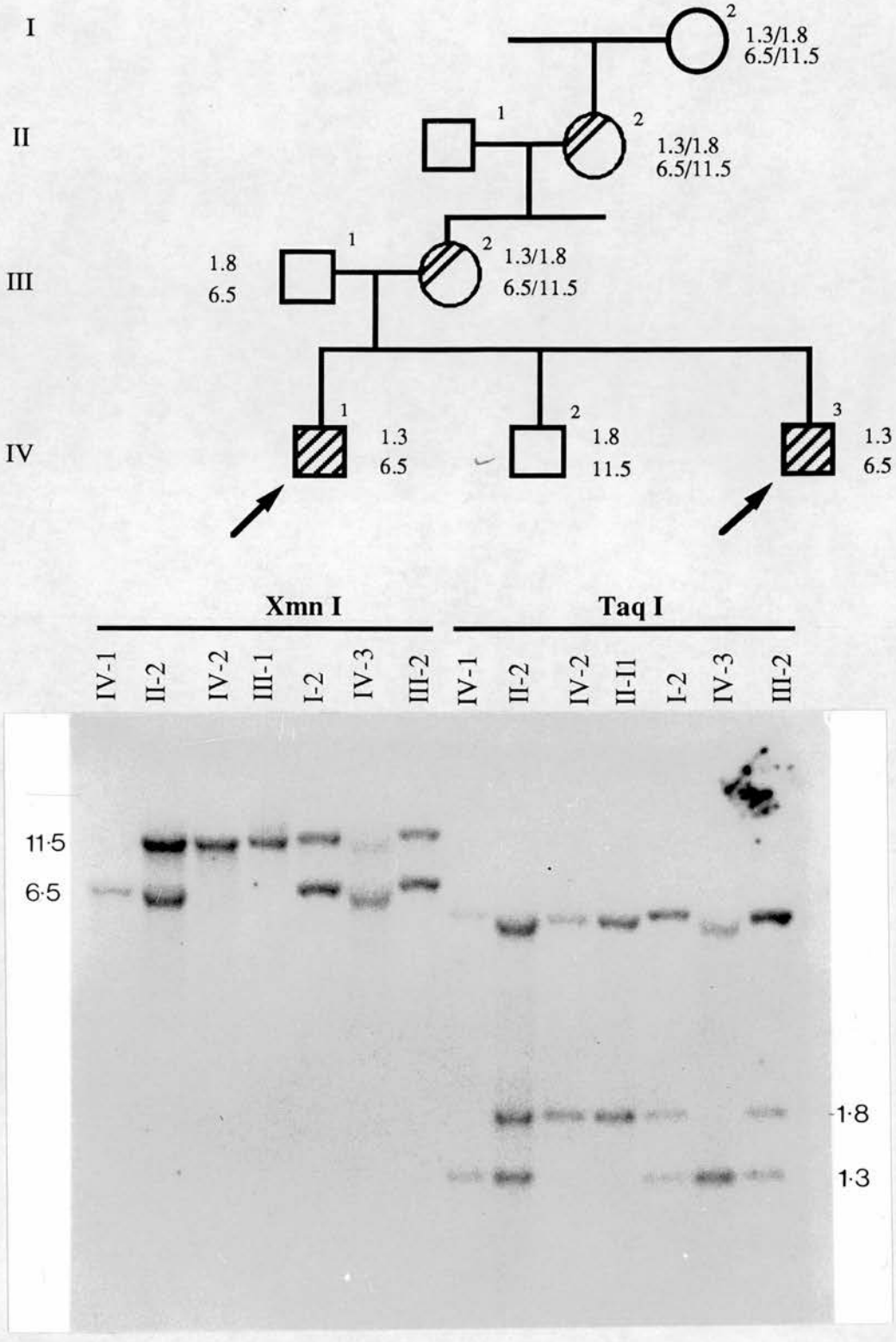


Figure 25. Autoradiograph of leucocyte DNA digested with Taq I and Xmn I and hybridised with the genomic FIX probe, Probe VIII. The polymorphic fragments at 1.3kb/1.8kb (Taq I) and 6.5kb/11.5kb (Xmn I) are marked.

2. Dde I polymorphism.

The Dde I polymorphism is situated 5' of exon B in the FIX gene and results in two allelic forms differing by 50bp of inserted DNA sequence (1.70kb and 1.75kb). Figure 26 shows Pedigree No. B2 (Appendix 2) in which the Taq I and Xmn I polymorphisms were unhelpful but the Dde I polymorphism proved informative.

In this family the mutant gene is marked by the 1.75kb allele of the Dde I polymorphism (III-1). From the pedigree, III-3 and II-3 are both potential carriers and both have an abnormal coagulation phenotype. The finding of low IX:C and IX:Ag values in these two individuals suggests that II-3 is likely to be a carrier and that the mutation in III-1 is not unique. If II-3 is a carrier then her daughter (III-3), having inherited the abnormal haplotype is also a carrier, a suggestion supported by her phenotype.

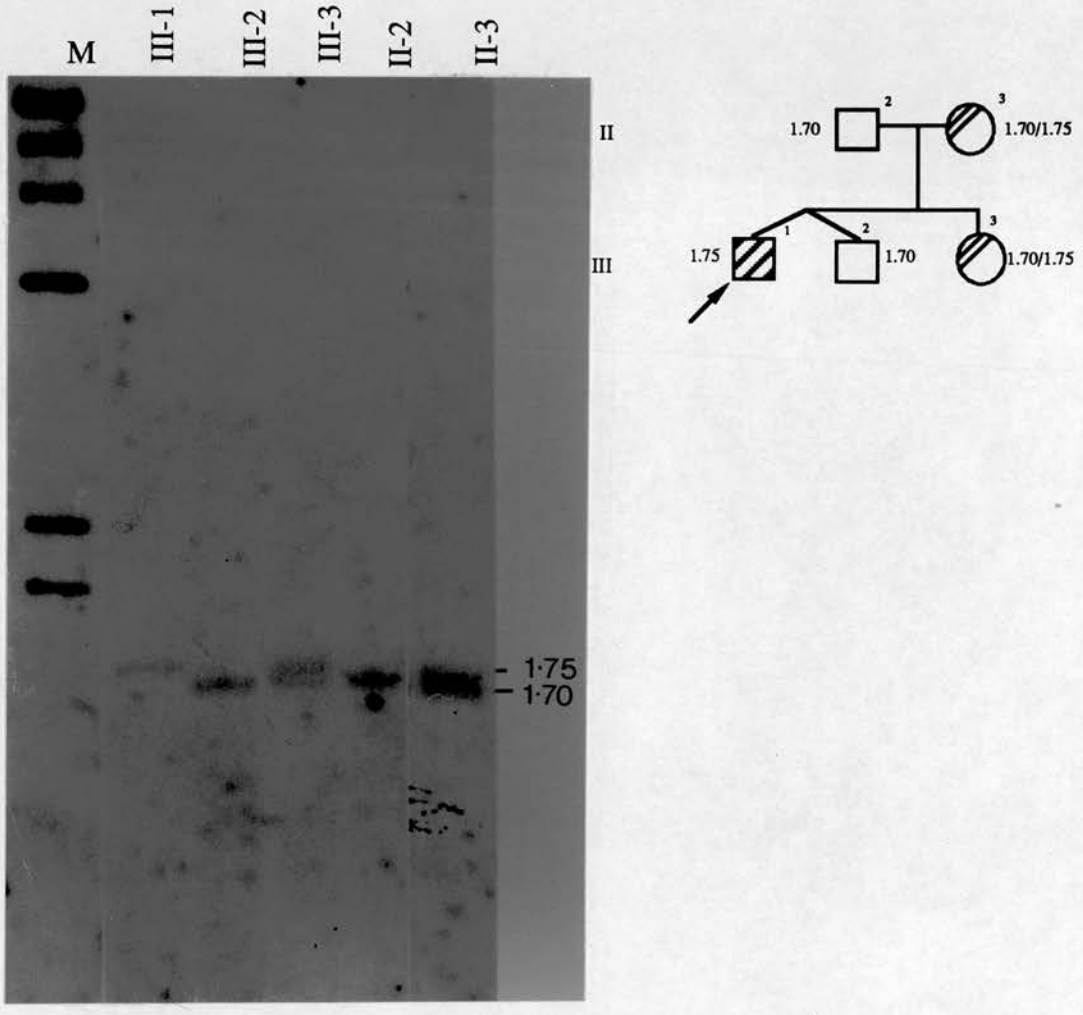


Figure 26. Autoradiograph of leucocyte DNA digested with Dde I and hybridised with the genomic Factor IX probe, Probe XIII. The polymorphic fragments at 1.70kb and 1.75kb are marked. M - Lambda DNA/Hind III markers.

3.6.3. Frequency of Intragenic Polymorphisms.

The frequencies of the allelic DNA fragments and the number of heterozygous females for the three intragenic polymorphisms are summarised in Table 32. This shows the data obtained for all women, unrelated females and the 3 obligate carriers.

The Taq I and Xmn I polymorphisms were informative in 9/11 women studied.
The Dde I polymorphism was only analysed in a single family but was informative in the 2 'at-risk' females.

Table 32. Allelic frequencies for the three intragenic polymorphisms used for the carrier detection studies in families with haemophilia B.

				Frequency of heterozygosity					
		Allele		All females		Unrelated females		Obligate carriers	
RFLP	No. X's analysed	Size (kb)	Freq.	No.	%	No.	%	No.	%
Taq I	33	1.3 1.8	0.394 0.606	9/11	81.8	3/4	75	3/3	100
Xmn I	33	6.5 11.5	0.394 0.606	9/11	81.8	3/4	75	3/3	100
Dde I	7	1.70 1.75	0.571 0.429	2/2	100	1/2	50	-	-

NB. 1. The predicted heterozygosity frequencies for the Taq I, Xmn I and Dde I polymorphisms are 47.7%, 47.7% and 49% respectively.

2. See Chapter 1 for published heterozygosity frequencies for the 3 intragenic polymorphisms.

3.6.4. Genotypic Analysis in Potential and Obligatory Carriers of Haemophilia B.

RFLP analysis was attempted in 8 potential carriers and 3 obligatory carriers of haemophilia B.

1. Potential carriers: of the 8 potential carriers, the coagulation phenotype was normal in 1, abnormal in 3 and in the remaining 4 women no data was available. The analysis of the potential carriers and the contribution from genotypic analysis is shown in Table 33 below. In 2 of 3 potential carriers with abnormal phenotypes, genotypic analysis confirmed them as carriers. However, genotypic analysis was unhelpful in the single potential carrier with a normal coagulation phenotype, in the four potential carriers in whom no coagulation data was available and in one of the 'at-risk' females in whom the coagulation phenotype was abnormal.

Table 33. Potential carriers of haemophilia B classified by coagulation phenotype and the results of genotypic and combined genotypic/phenotypic analysis.

	Carrier status following genotypic analysis			Carrier status following combined phenotypic and genotypic analysis		
Phenotype	**Carrier**	**Normal**	**Non-informative**	**Carrier**	**Normal**	**Non-informative**
Normal (n=1)	-	-	1	-	-	1
Abnormal (n=3)	-	-	3	2	-	1
No Data (n=4)	-	-	4	-	-	4

2. Obligate carriers: of the 3 obligatory carriers identified, 2 had abnormal coagulation phenotypes; all 3 were heterozygous for both the Taq I and Xmn I polymorphisms.

Overall, genotypic analysis was helpful in establishing the carrier status of 2 potential carriers and demonstrating informative polymorphisms for use in prenatal diagnosis in 3

obligatory carriers. The 2 potential carriers classified as carriers were from the same pedigree (B2) whilst in the remaining 6 potential carriers whose carrier status could not be definitively established, 4 came from a single pedigree (B4) in which it was impossible to establish the origin of the mutation and, therefore, to track it by RFLP analysis.

In 4 women in whom no coagulation data was available, measurement of their IX:C may prove useful establishing their risk of carriership.

3.6.5. Origin of the Mutant Gene in Families with Spontaneous Haemophilia B.

With the pedigree data available, it was not possible to establish the origin of the mutant FIX gene in any of the kindred with sporadic disease. In Pedigree No. B2 (Appendix 2) although II-3 was identified as a probable carrier from a combination of phenotypic and genotypic analysis, it was not possible to establish whether the abnormal gene had arisen with her or in a previous generation.

3.7. Antenatal Diagnosis.

The availability of antenatal diagnosis for both obligatory and potential carriers was established (Table 37). All of the obligate carriers and the two potential carriers shown genotypically to be carriers, were informative for one of the intragenic polymorphisms and could, therefore, be offered accurate and early prenatal diagnosis with chorionic villus sampling.

In the case of the remaining potential carriers, although they were all informative for one or more polymorphic markers, their carrier status was not established. It would be feasible to offer antenatal diagnosis using RFLP analysis and in those cases in which a male fetus had inherited the same haplotype as an affected male, to perform fetal blood sampling. If RFLP analysis indicated a male fetus had inherited the other haplotype, the

pregnancy could be allowed to continue safely. Although this provides a solution, the detection of kindred specific mutations allows more accurate carrier detection, prenatal diagnosis and genetic counselling.

3.8. Pedigree No. B5 (Appendix 2).

A single female was identified within this study with low levels of both IX:C (0.16iu/dl) and IX:Ag (0.12u/dl) but no family history of Christmas disease or of any other bleeding diathesis. However, she had a life-long history of recurrent bleeding associated with minor trauma and surgery but denied any spontaneous bleeding episodes. Coagulation studies on her daughter were entirely normal.

In view of these findings the possibility of an unusual chromosomal karyotype was considered. Karyotypic analysis demonstrated a mosaic picture with the existence of two clones:

i. A major clone (85%) with one normal X and one abnormal X chromosome (see Figure 27).

ii. A minor clone (15%) which had only one normal X chromosome (see Figure 28).

The chromosomal rearrangement of the major clone was interpreted as inversion X(q24-26) - a linear rearrangement with no loss of genetic material at the level of light microscopy.

There are a number of possibilities to explain the phenotypic and karyotypic findings:

a) The chromosomal inversion may involve the FIX gene resulting in only a single functional gene. Extreme Lyonisation of the other X-chromosome could result in reduced expression and therefore, low FIX levels.

b) The inversion although close to the FIX gene by cytogenetic analysis, at a molecular

level may lie many millions of bases away from it and the minor clone is the important abnormality. This clone may involve the hepatocytes such that a proportion of the cells have only a single functional X chromosome. If this single X-chromosome carried a mutant gene the cells would produce only defective factor IX.

c) A mutation in one of the FIX genes may occur leading to non-expression of that gene. Extreme Lyonisation could then result in reduced expression of the remaining FIX gene.

In an attempt to establish whether there was a mutation in the FIX gene, DNA from the proposita was isolated from peripheral blood leucocytes. Restriction mapping of the gene was performed with BamH I, Hind III, EcoR I, Taq I and the FIX cDNA as a probe.
Taq I was used as its recognition sequence (TCGA) occurs at a recognised 'hotspot' (CpG dinucleotide) for mutation in the human genome and variations at this site will result in an altered Taq I map. The remaining enzymes cut commonly throughout the human genome and it was hoped that a major insertion/deletion within the FIX gene would alter the pattern of fragments generated. However, in each case a pattern identical to that of a normal control DNA was seen, indicating that no major rearrangements within the gene had occurred although not excluding a small deletion, insertion or point mutation.

Further analysis of the FIX genes in this individual was kindly performed by Prof. F. Giannelli at Guy's Hospital, London. Using the PCR technique the entire coding region, intron-exon splice sites and the promoter region of the FIX gene was amplified and sequenced. This demonstrated a single point mutation (GGA-GTA) resulting in the substitution of a Glycine by a Valine at position 76. This region, encoded by exon D (nucleotides 10392-10505) forms part of the connecting region of the FIX protein. Glycine is the simplest of the amino acids having no side chain whilst valine is larger and contains 2 methyl group side-chains. Although both are neutral amino acids at

physiological pH, it is possible that this substitution is sufficient to cause a perturbation in the FIX molecule resulting in a failure in secretion or its increased removal from the plasma.

Although a mutation has been demonstrated within a FIX gene, it is still unclear what is happening at the hepatocyte level. The aetiology of the disease in this lady, therefore, remains speculative. The finding of a normal karyotype and coagulation phenotype in the daughter of the propositus does not exclude her as a carrier and characterisation of her FIX gene is needed to establish her carrier status.

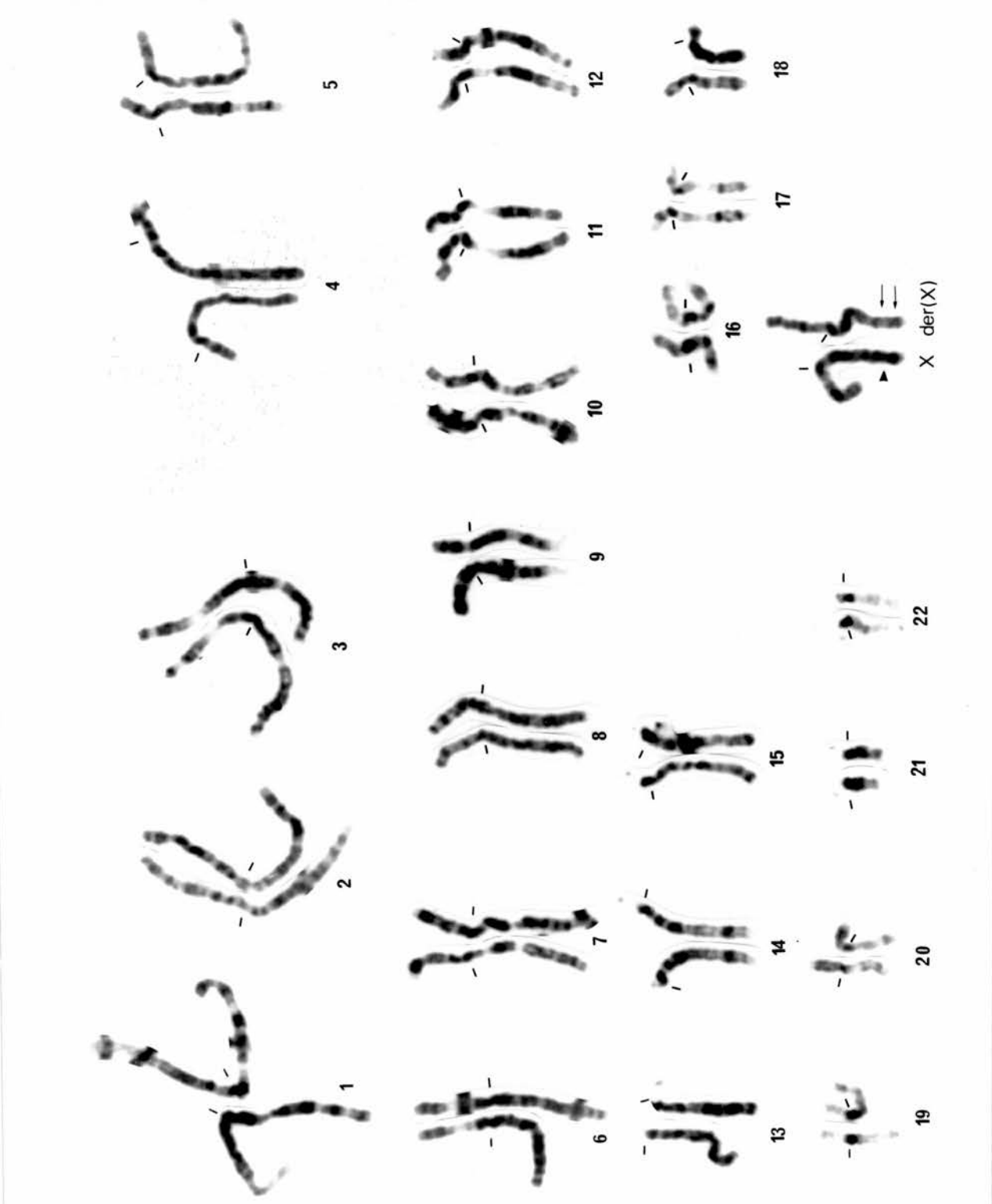


Figure 27. Karyotype analysis on the propositus from Pedigree No. B5 showing the major clone demonstrating the inversion on one X chromosome that has occurred in the region of the FIX gene.

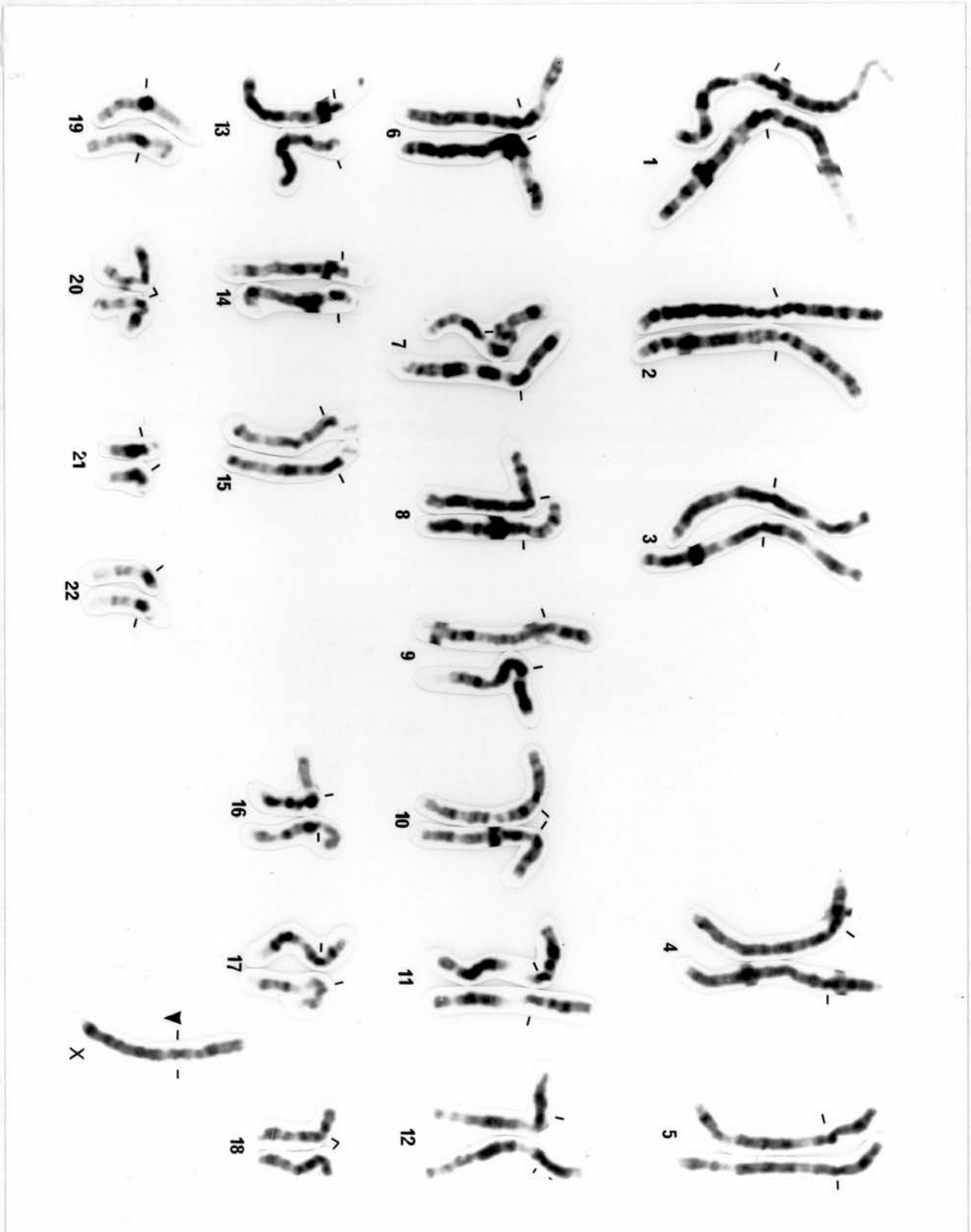


Figure 28. Karyotype analysis on the propositus from Pedigree B5 showing the minor clone with only a single X-chromosome.

3.9. The use of FVIII gene probes in X-linked adrenoleukodystrophy (Pedigree No. C1 - Appendix 3).

During this study, help was sought to clarify the carrier status of a female in a family with X-linked adrenoleukodystrophy (ALD). There was no history of haemophilia A or B. X-linked ALD is characterised by the progressive accumulation of very long chain fatty acids (VLCFA) in neural white matter, adrenal glands, fibroblasts and plasma leading to progressive demyelination and adrenal insufficiency with features resembling Addison's disease.

The gene for ALD maps close to that of the FVIII gene at Xq28,[337,338,339,340] and has been shown to be tightly linked to both the G6PD[337] and DXS52[339] loci. Auborg and co-workers analysed 7 families with ALD using the ST14(DXS52) marker. The Lod score for this data was 13.766 at a maximum likelihood estimate of recombination (θ) of 0%. No evidence for recombination between the ALD and ST14(DXS52) loci was found (confidence limits 0-4%). The use of the ST14 marker may, therefore, be useful in carrier detection studies in ALD.

Within this kindred III-1 is an affected male and both clinically and biochemically it was felt that III-2, his brother, was also affected. Their mother, therefore, is an obligatory carrier of the disease. Information was sought as to whether her sister II-4 might be a carrier. The two linked Factor VIII polymorphisms - Bgl II (DX13) and Taq I, (ST14) together with the three intragenic polymorphisms were used in an attempt to track the mutant gene within the family. The family is non-informative for the three intragenic probes but informative for the Bgl II marker which suggests that II-4 is a carrier. However, analysis with the ST14 probe showed a recombination has taken place between the two linked markers (see Pedigree/genotype data - Appendix 3). However as the ST14 (DXS52) probe is the closest to the adrenoleukodystrophy gene the results reduce the risk that that II-4 is a carrier. Ideally, further linkage studies with probes closer to or within the gene are required.

3.10. Family Pedigree Data.

1. Summary of Pedigree Data for Haemophilia A and B (Tables 34-37).

Tables 34-37 summarise the pedigree, phenotypic and genotypic data from the 44 families with haemophilia A and the 5 families with haemophilia B studied in this Thesis.

2. Family Pedigrees (Appendices 1-3).

The family pedigrees and individual results obtained from the phenotypic and genotypic analyses are contained in the Appendices 1-3. Two consecutive pages are devoted to each kindred. The first page shows the full pedigree together with tabulated data from the phenotypic and genotypic analyses. The pedigree is based exclusively on family data and does not include any of the results from the carrier detection studies. For each female their carrier status derived from the pedigree from coagulation testing and finally from genotypic analysis is shown. The second page contains a limited pedigree showing only those family members in which genotypic analyses were performed. The haplotype for each individual is plotted on the family tree. The results from both pages are summarised and the value of the cumulative data analysed in terms of the availability of carrier detection and of antenatal diagnosis. Comments for each family highlighting particularly interesting or unusual findings are included as a separate section at the bottom of the second page.

Appendix 1 contains pedigree data for the haemophilia A families, Appendix 2 for the haemophilia B families and Appendix 3 for the non-haemophilia families.

Table 34. Summary of pedigree data for haemophilia A.

Ped No.	Gens. studied	Males studied		Obligate carriers Phenotype		Potential Carriers Phenotype		Phenotype + Genotype		Availability of antenatal diagnosis		
		Affected	Normal	Normal	Abnormal	Potential	Definite	Definite	Potential	Geno.	Geno/ FBS	FBS
Family history of haemophilia A (>1 generation).												
1	2	1	3	-	1	-	-	-	-	-	1	-
3	2	1	-	1	1	-	-	-	-	-	2	-
4	2	2	4	-	1	2	-	2N	-	1	-	-
6	2	1	-	-	1	-	-	-	-	1	-	-
8	2	1	1	1	-	1	-	-	1	-	-	1
10	3	2	1	3	-	-	-	-	-	2	1	-
13	2	1	1	-	1	1ND	-	1C	-	1	-	1
15	2	1	-	-	-	1	2	-	3	-	2	1
16	3	-	4	-	1	2	1	2N 1C	-	2	-	-
22	4	1	1	-	4	-	1	-	1	5	-	-
23	3	2	-	-	2	-	-	-	-	1	-	1
26	3	1	2	1	1	5	-	5N	-	2	-	-
27	3	2	3	1	1	1	2	2N 1C	-	3	-	-
28	3	1	-	-	3	-	-	-	-	1	1	1
30	2	1	1	-	1	-	1	1C	-	1	1	-
34	2	2	3	-	1	1	-	1C	-	1	1	-
35	2	1	1	1	1	-	-	-	-	1	1	-
36	2	1	-	2ND	-	-	-	-	-	-	-	2
38	4	2	1	1	1	3 2ND	1	4N	2	2	1	1
39	2	2	-	1	1	1	-	-	1	-	2	1
41	2	1	1	-	1	1	-	1N	-	1	-	-
44	2	1	1	1OC ND		1ND	-	1C	-	-	2	-
Family history of haemophilia A (1 generation).												
7	2	2	1	-	1	-	1	1C	-	-	2	-
12	3	2	2	1	-	-	1	1N	-	-	-	1
24	2	2	1	-	1	-	-	NA	NA	-	1	-
Family history unknown.												
29	2	2	1	1	-	-	-	NA	NA	1	-	-

Table 35. Summary of pedigree data for haemophilia A (sporadic disease).

Ped No.	Gens. studied	Males studied		Carrier status of potential carriers by Phenotype		Phenotype + Genotype		Availability of antental diagnosis		
		Affected	Normal	Potential	Definite	Definite	Potential	Geno.	Geno/ FBS	FBS
Spontaneous disease.										
2	3	1	-	2	-	-	2	-	2	-
5	2	1	2	1	-	1N	-	-	-	-
9	3	1	2	3	1	2N 1C	1	1	1	-
11	3	1	1	3	1	2N 1C	1	1	1	-
14	2	1	1	1	-	-	1	-	1	-
17	3	1	1	3	-	1N	2	-	2	-
18	2	1	1	1	-	-	1	-	1	-
19	2	1	1	-	3	3C*	-	2	-	1
20	3	1	1	1	2	3N	-	-	-	-
21	3	1	1	-	3	-	3*	-	3	-
25	2	1	-	2	2	-	4*	-	2	2
31	3	1	2	3	1	3C 1N	-	2	1	-
32	3	1	1	2	1	-	3*	-	1	2
33	3	1	2	4	-	1N	3	-	1	1
37	2	1	1	2	-	1N	1	-	1	-
40	2	1	1	1	1	1N	1	-	1	-
42*	2	-	2	-	2	2C	-	1	-	1
43	2	1	1	2ND	-	1N	1	-	1	-

* see pedigree.
NA - not applicable; C - carrier; N - normal; OC - obligate carrier; PC - potential carrier;
Ped. No. - Pedigree number; Gens. studied - number of generations available in each kindred for analysis; AND - antenatal diagnosis; Gen - genotype; FBS - fetal blood sampling; ND - no data.

Table 36. Summary of pedigree, phenotypic and genotypic data from 44 families with haemophilia A.

Number of women available for study													
		Obligatory carriers			Potential Carriers								
Males studied		Phenotyic analysis			Phenotypic analysis			Phenotypic+Genotypic analysis			Availability of antenatal diagnosis		
Affected	Normal	Normal	Abnormal	No Data	Normal	Abnormal	No data	Carrier	Normal	Potential Carrier	Geno.	Geno/ FBS	FBS
Family history of haemophilia (>1 generation) (n=22).													
28	28	10	23	3	18	8	4	6	16	8	25	15	10
Family history of haemophilia (1 generation) (n=3).													
6	4	1	2	-	-	2	-	1	1	-	-	3	1
Family history unknown (n=1).													
2	1	-	1	-	-	-	-	-	-	-	1	-	-
Spontaneous disease (n=18).													
17	21	-	-	-	29	17	2	10	14	24	7	21	6
Overall summary (n=44).													
53	54	11	26	3	47	27	6	17	31	32	33	39	17

NB. 5 normal females identified from pedigree analysis are not included in this summary.
Geno. - genotype; Geno/FBS - Genotypic analysis + fetal blood sampling; FBS - fetal blood sampling.

Table 37. Summary of pedigree, phenotypic and genotypic data from 5 families with haemophilia B.

		Number of women available for study											
		Obligatory carriers				Potential Carriers						Availability of antenatal diagnosis	
		Males studied		Phenotypic analysis		Phenotypic analysis			Phenotypic + Genotype analysis				
Ped. No.	Gens. studied	Affected	Normal	Normal	Abnormal	Normal	Abnormal	No data	Carrier	Normal	Potential carrier	Geno.	Geno/ FBS
Family history of haemophilia B (n=2).													
B1	2	1	-	1	-	-	-	-	-	-	-	1	-
B3	4	2	3	-	2	-	-	1	-	-	1	2	1
Spontaneous disease (n=3).													
B2	2	1	2	-	-	-	2	-	2	-	-	2	-
B4	2	1	1	- -	- -	1	1	3	-	-	5	-	5
B5*	1	-	-	-	-	-	'1'	-	1	-	-	1	-
Overall summary (n=5).													
		5	6	1	2	1	4	4	3	-	6	6	6

* see pedigree.
Ped. No. - Pedigree number; Gens. studied - number of generations available in each kindred for analysis; Geno. - genotype; Geno/FBS - genotype + fetal blood sampling.

3.11. Summary of Results.

The results of the phenotypic and genotypic studies in haemophilia A and B are summarised in Figures 29 and 30.

3.11.1. Haemophilia A.

125 females with haemophilia A were studied. From pedigree analysis 40 were identified as obligate carriers, 80 as potential carriers and 5 women were considered normal.

A. Obligate carriers: of the 40 obligate carriers, 26 were shown to have an abnormal coagulation phenotype, 11 to have a normal phenotype and in 3 no coagulation data was available. Genotypic analysis established 23 as being informative for one or more of the intragenic polymorphisms and 15 as informative for a linked polymorphism. Two women were non-informative although the multi-allelic, extragenic Taq I RFLP was not analysed.

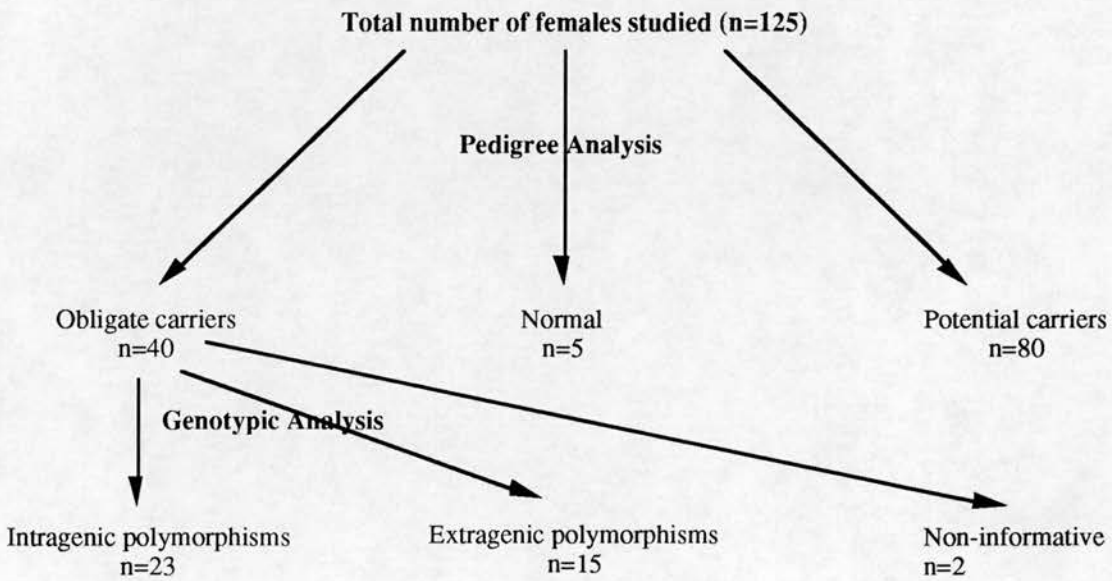
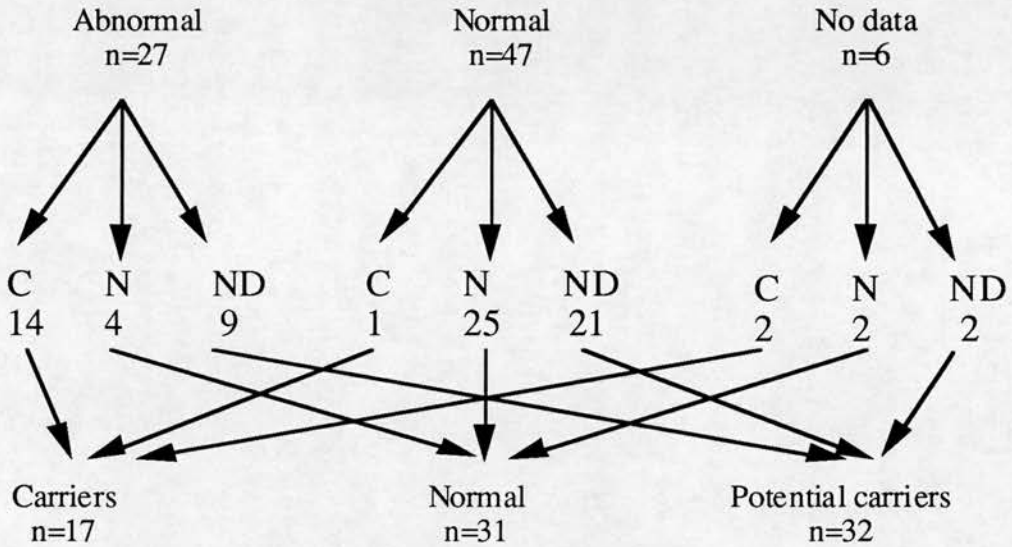


Figure 29. Summary of data following phenotypic and genotypic analyses in 125 women from 44 families with haemophilia A.

B. Potential carriers: of the 80 potential carriers identified within the study, 27 had an abnormal coagulation phenotype, 47 were normal and in 6 no coagulation data was available. Combined phenotypic/genotypic analysis classified 17 of the 80 potential carriers as carriers, 31 as normal but in 32 women genotypic analysis could not establish their carrier status and they, therefore, remain potential carriers.

Results from phenotypc analysis of 80 potential carriers of Haemophilia A



Results from combined phenotypic/genotypic analysis of 80 potential carriers of Haemophilia A

Figure 30. Results following phenotypic and combined phenotypic/genotypic analysis in 80 potential carriers of haemophilia A. (C - carrier; N - normal; ND - no data).

3.11.2. Haemophilia B.

11 females with haemophilia B (Christmas disease) from 4 families were studied. A single female with an unusual karyotype was also identified as having haemophilia B. From pedigree analysis, 3 of the women were identified as obligate carriers and 8 as potential carriers. Phenotypic data was available on 3 of the obligate carriers and 4 of the potential carriers. Phenotypic analysis showed 2 of 3 obligate carriers to have a low IX:C and 3 of 4 potential carriers.

Following combined phenotypic/genotypic analysis 2 of 8 potential carriers were identified as carriers but in the remainder accurate classification was not possible. All 3 of the obligate carriers were informative for one or more of the intragenic polymorphisms.

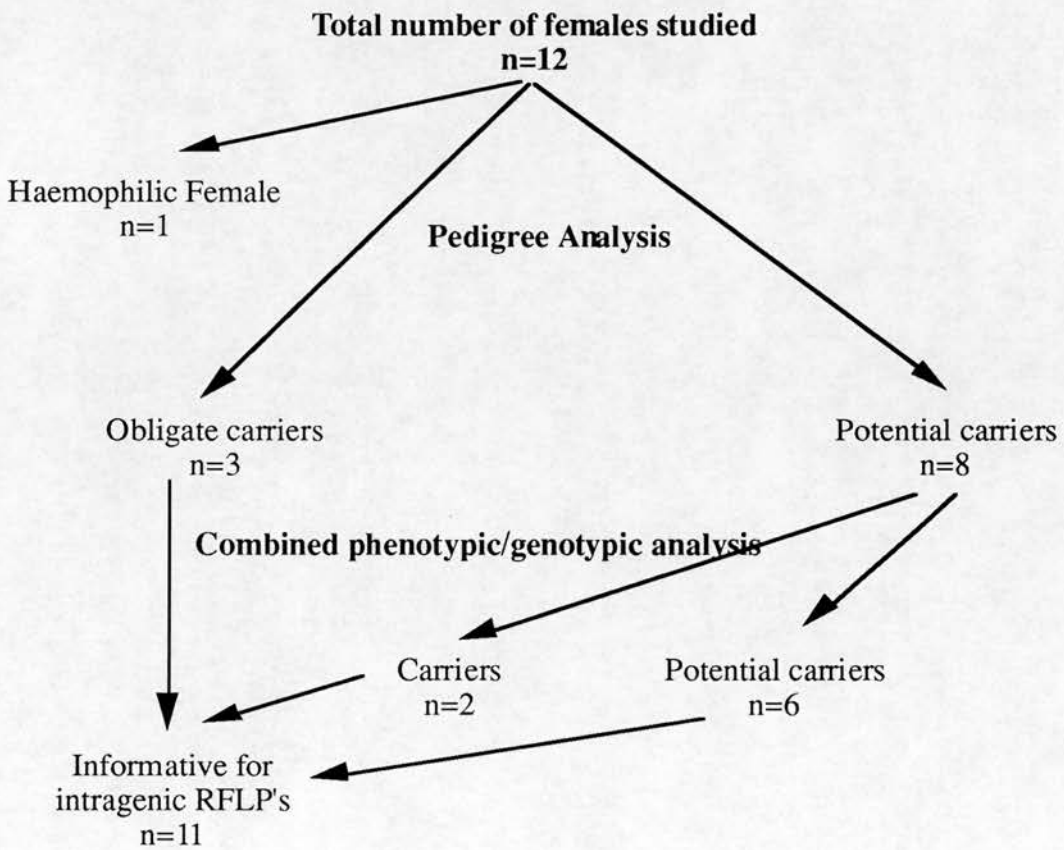


Figure 30. Summary of data from phenotypic and combined phenotypic/genotypic analysis in 12 women from 5 families with haemophilia B (Christmas disease).

Chapter 4 - Discussion.

Discussion.

Over the past 20 years the development of better standardisation of functional assays (VIII:C, vWF activity), the introduction of immunological assays (VIII:Ag and vWF:Ag) and the application of molecular biology techniques has improved the ability to discriminate between carriers of haemophilia A and normal women.

Advances in molecular biology have now made the characterisation of kindred specific defects in families with haemophilia B a real possibility but the size of the FVIII gene has precluded such an approach until very recently and RFLP analysis has, therefore, remained a major technique for both carrier detection and prenatal diagnosis in haemophilia A.

This Thesis addresses the role of coagulation testing and restriction fragment length polymorphism (RFLP) analysis in establishing carriership in haemophilia A and B and how best to provide prenatal diagnosis to 'at-risk' women.

In this Chapter the results from pedigree, phenotypic and genotypic analysis presented in the previous chapter will be discussed in the context of previously published studies.

Analysis of the family pedigree forms an essential part of carrier detection in families with a history of a bleeding diathesis and the pattern of inheritance eg. autosomal, X-linked, dominant or recessive, may suggest specific diseases. Within this study a female could be classified by pedigree analysis as either an obligate carrier, a potential carrier or normal.

In some instances, pedigree analysis may allow a female to be excluded as a carrier eg. the daughter of a normal male in a family with haemophilia. A number of women identified as obligate carriers had failed to appreciate the importance of being the

daughter of a haemophiliac. This lack of knowledge emphasises the importance of good genetic counselling in families with any genetic disorder. However, for many women pedigree analysis can only establish them as potential carriers and further testing is required to define their carrier status.

Coagulation testing and phenotypic analysis in families with haemophilia is undertaken for a number of reasons:

1. To identify within a kindred which males are affected.
2. To identify any women, either potential carriers or obligate carriers with low VIII:C values and, therefore, at increased risk of haemorrhage.
3. To try and define the carrier status of potential carriers.
4. As an aid to the interpretation of genotypic analysis.

In the carrier detection studies reported in this Thesis, measurement of the coagulation factors and relevant immunological assays were used to estimate the risk of carriership in individual females. These results were then compared to those obtained from genotypic analyses.

Measuring VIII:C and vWF by immunological and biological functional assays to compare values between obligate carriers and normal women has, until recently been the principle method for carrier detection in haemophilia A. By applying various discriminant functions to potential carriers, the likelihood of carriership in a particular individual can be established although for many, carriership cannot be excluded. Published studies, reviewed in the Introduction (Chapter 1) to this Thesis have shown that the measurement of the ratio of VIII:C to vWF:Ag (and possibly VIII:C to vWF:RCo) are superior to the measurement of VIII:C alone in the detection of haemophilia A carriers.

In this Thesis, normal ranges for both obligate and normal females were established using age-matched controls from which the VIII:C/vWF:Ag and VIII:C/vWF:RCo ratios were derived which correctly classified all of the normal women. These ratios were then used to analyse both the obligatory carriers and the potential carriers identified within the study.

The mean VIII:C, vWF:Ag and vWF:RCo values obtained for the control groups were similar to those reported by other workers (reviewed in Chapter 1) and no differences were found between vWF:Ag and vWF:RCo values for each group contrary to the results of Zimmerman et al who found vWF:Ag assays to be higher in carriers.[255] In addition, no differences were found between women taking the oral contraceptive pill and those who were not although this has been reported to cause elevations in both VIII:C and vWF.[247]

The VIII:C/vWF:Ag ratio appeared to be a more sensitive discriminant of carriership than the VIII:C/vWF:RCo ratio as it correctly classified 65.2% of the control obligate carriers and 56.8% of the study obligate carriers whilst the VIII:C/vWF:RCo ratio correctly classified only 21.7% of the control obligate carriers and 43.4% of the study obligate carriers. Our findings that the VIII:C/vWF:Ag ratio correctly classified 65% of the control obligatory carriers and only 56.8% of the study obligatory carriers are inferior to many other studies. It is likely that this reflects, to some extent, the method by which the coagulation data was analysed. A number of studies have reported that the percentage of obligatory carriers correctly classified by measuring the VIII:C/vWF:Ag ratio ranges from 70-100% (Chapter 1). In 1986 a Multicentre International Study[299] concluded that the inclusion of both age and ABO blood group together with the minimisation of laboratory assay errors improved the discrimination between normal women and obligatory carriers but that assays other than VIII:C and vWF:Ag had no effect. Earlier studies have shown that repeated testing can result in better discrimination between carriers and non-

carriers.[296]

Because many of the families in this study live some considerable distance from the Centre (both within and outwith the West Midlands Health region) it was only possible to undertake coagulation factor assay on a single occasion when samples for DNA isolation were obtained. Because of this statistical analysis has not been applied to carrier prediction of the study group but the coagulation phenotype has been used as an indication of carriership and as an aid to the interpretation of the results from genotypic analysis.

Only 5 families with haemophilia B consented to be studied. The mean IX:C assays in both the obligate and potential carriers (in whom data was available) was lower than that of 15 normal women included for comparison but the variation in both groups was large and there was considerable overlap in results suggesting that carrier detection using IX:C alone is a poor discriminant between normal women and obligatory carriers. Two of the three obligate carriers and three of the four potential carriers had IX:C levels below that of the 15 normal female controls and would, therefore be classified as carriers. However, the numbers in both these groups are small and the conclusions which can be drawn are limited. The measurement of IX:Ag has been suggested as a method for increasing the accuracy of carrier detection either alone or in combination with measurements of IX:C.[310,314,315] The measurement of both IX:C and IX:Ag also allows any CRM^+ families to be identified. No CRM^+ families were detected with IX:Ag testing but insufficient IX:Ag data was available to determine the efficacy of this assay as a discriminant in carrier detection.

The method employed to predict carriership is open to major criticism. Although the two control groups (normal women and obligatory carriers) were matched (for age and contraceptive usage) and the coagulation data carefully collected, the assignment of an arbitrary 'cut-off' value to predict carrier status has major limitations. Such an approach

is very dependent upon sample size and it is probable that increasing the size of the two control groups would result in an entirely different 'cut-off' value and, therefore, the assignment of carriership in the 'at-risk' women. A more logical approach would be to use Bayesian analysis - this would take into account pedigree, phenotypic and genotypic information, assigning a final risk of 'carrier' or 'non-carrier' to all 'at-risk' women. Such an approach has been reviewed in Chapter 1 of this Thesis.

Recent advances in molecular biology eg. PCR, chemical cleavage of mismatches, single-strand conformation polymorphism analysis, have made the characterisation of individual mutations more feasible than with previous technology. For haemophilia B the identification of kindred specific mutations is now possible and this should improve both the accuracy and the availability of carrier detection. However, in haemophilia A the size of the FVIII gene (186kb) has made, until very recently, the identification of individual mutations virtually impossible for all but a small number of individuals. When such mutations have been characterised it is generally because they have occurred at restriction enzyme recognition (eg. Taq I) sites known to occur at 'hotspots' (eg. CpG dinucleotides) for mutation in the human genome,[194,231,341] or are the result of major gene deletions.

More recently the PCR technique has been used to amplify the small amounts of FVIII mRNA produced at non-hepatic sites in haemophiliacs[342,343,344] and this may prove to be an invaluable approach for identifying mutations in which an abnormal mRNA is produced. A concern is that some mutations within the FVIII gene arise as a failure of transcription and, therefore, will not be detected using this technique. In addition, a recent report indicates that approximately 50% of mutations in severe haemophilia A are not within the coding regions, the splice junctions, the promoter region or the polyadenylation region of the factor VIII gene,[211] and are unlikely, therefore, to be associated with the production of an abnormal ectopic FVIII mRNA.

The size of the factor IX gene has meant that the detection of kindred-specific mutations is more feasible and with current techniques, direct carrier detection by identification of the presence or absence of specific mutations should be possible in most cases of haemophilia B.[242]

At present, therefore, the analysis of RFLP's remains an important part of carrier detection in haemophilia A but to a lesser extent with haemophilia B.

The usefulness of RFLP's in carrier detection is related to their frequency within a population. A number of the RFLP's used in carrier detection studies in haemophilia A show racial segregation and the population studied is, therefore important. The families studied in this Thesis were mainly European with a small number of Asian families and therefore, polymorphisms shown to be useful in other races were not analysed.

Three intragenic - Bcl I, Xba I and Bgl I and two linked polymorphisms - Bgl II and Taq I were used for genotypic studies in haemophilia A. The Bcl I polymorphism was studied in most women and is now the first of the RFLP's to be analysed as this can be readily performed by PCR. Gitschier et al[193] predicted that 42% of women would be heterozygous for this polymorphism - in our study an identical figure (41.8%) was observed although in unrelated women the number of heterozygotes was less at 36.5% and similar to that found in obligatory carriers (33%). A number of other studies[334,335,336,345] have reported similar heterozygosity frequencies and our findings confirm the value of this polymorphism in carrier detection studies.

In women in whom the Bcl I polymorphism was non-informative, the intragenic Xba I polymorphism was analysed. In initial studies, analyses were performed by Southern blotting but in the latter part of the study, a combination of PCR and Southern blotting was used. Wion et al[195] predicted from the frequencies of the two alleles that 48% of

women would be heterozygous for this marker. In our study, a slightly higher frequency (57.1% in related women, 55.5% in unrelated women and 58% in unrelated obligate carriers) was observed indicating that the Xba I polymorphism should be a useful marker in carrier detection studies. A number of other studies have found similar heterozygosity frequencies[334,336] confirming the usefulness of this polymorphism.

In the 45% of women in whom the Bcl I polymorphism was non-informative, 52% (of unrelated women) were heterozygous for the Xba I polymorphism. Therefore, a combination of these two intragenic polymorphisms will be informative in 78% of women.

The Xba I and Bcl I polymorphisms are situated close to each other within the FVIII gene and analysis of the haplotype frequencies shows that of 40 unrelated women the 1.1/4.8kb haplotype was not observed. From the allelic frequencies this haplotype should be found in 12.5% of cases and these findings are consistent with the two polymorphisms exhibiting a degree of linkage disequilibrium similar to that originally reported by Wion et al. Moodie reported less linkage disequilibrium between these two sites and specifically a frequency for the 1.1/4.8kb haplotype of 10%. This has not been confirmed by this study or by that of Wion who also failed to identify this particular haplotype in any individual. In addition our study has shown an increased frequency for the 0.8/4.8kb haplotype and a reduced frequency for the 0.8/6.2kb haplotype. The explanation for these differences is likely to reflect variations in allelic frequencies between differing populations.

In addition to the Xba I polymorphism reported by Wion et al, a number of additional polymorphisms recognised by the same probe (p482.6) have been reported.[346,347,348] Two of these are intragenic[346] and in a Chinese population (as originally reported) the frequencies of heterozygotes for the Xba I sites are 0.49 (the site originally described by

Wion et al), 0.18 and 0.30. The third Xba I polymorphism is extragenic[347] and located in the area of the X chromosome homologous to that region of the FVIII gene detected by the p482.6 genomic FVIII probe. This region gives rise to the 6.6kb band during Southern blot analysis for the Xba I polymorphism and co-amplifies during PCR detection (generating a 96bp band). It has been mapped to a region within 1.5Mbp of the FVIII gene at DXS115.[349] The frequency of heterozygosity at this locus is reported as 13% in a German population.[347] The fourth polymorphism, also extragenic and detected using the intron 22 specific probe p482.6 is identified with BstX I and also maps to DXS115.[348] These additional polymorphisms will increase the number of heterozygous women but further work is required to accurately establish their allelic frequencies and the risk with the extragenic RFLP's of recombination.

In women in whom both the Bcl I and Xba I polymorphisms were non-informative, the intragenic Bgl I marker was analysed. Unfortunately the two alleles of this polymorphism occur in Europeans with markedly unequal frequencies (5.0kb allele 0.94; 20kb allele 0.06) suggesting that few women will be heterozygous for this marker. The frequencies for the two alleles found in this study are similar to those reported by Antonarakis et al[194] and others.[334] From the allelic frequencies it is predicted that 11.3% of women will be informative for this polymorphism, similar to the observed frequency of 13%. However, when those women who were heterozygous for the Bgl I polymorphism were analysed for heterozygosity at other sites, all women informative for the Bgl I marker were also informative for the Bcl I polymorphism suggesting strong linkage disequilibrium between the two sites. No women were identified who were homozygous for the Bcl I and Xba I polymorphisms but heterozygous for the Bgl I polymorphism. In American Blacks, the frequency of Bgl I heterozygotes approaches 40% making it an extremely useful marker and emphasising the importance of establishing allelic frequencies in local populations.

Analysis of the Xba I, Bcl I and Bgl I haplotypes shows that the several of the haplotypes occur more frequently than predicted from the allelic frequencies whilst others occur less frequently and indicates linkage disequilibrium. The finding of low heterozygosity frequency and linkage disequilibrium with the Bcl I and Xba I polymorphisms for the Bgl I polymorphism indicate that it has limited value in our local population. Analysis of the linked RFLP's (Bgl II and Taq I) would be more appropriate in women homozygous for the Bcl I and Xba I polymorphisms.

The DNA from women in whom the intragenic markers were non-informative was analysed using two polymorphisms tightly linked to the FVIII gene - Bgl II and Taq I both situated centromeric to the FVIII locus. The Bgl II polymorphism was the first RFLP to be reported and used for carrier detection in haemophilia A[190] and was the first for which a probe was available at the start of this study and, therefore, results are available on the majority of women. Harper reported a heterozygosity frequency of 50% in their population for the Bgl II RFLP and predicted a 9% recombination rate between the polymorphic site and the FVIII gene. Within this study the frequency of observed heterozygotes (58.9% all women, 67% unrelated women, 65% unrelated obligate carriers) is similar although slightly higher than that reported in previous studies[334,335,345] suggesting it will be a useful marker and in view of its distance from the previously identified intragenic polymorphisms, is unlikely to show significant linkage disequilibrium. Importantly, for unrelated women in whom both the Bcl I and Xba I polymorphisms were unhelpful, heterozygosity for the Bgl II polymorphism was found in 56% of cases. Therefore, 90% of women will be informative for one of the two intragenic polymorphisms or the linked Bgl II polymorphism.

In occasional women, homozygous for all the intragenic and the linked Bgl II polymorphisms, the multi-allelic Taq I marker was analysed. This polymorphism has at least 10 alleles, and as many as 90% of 'at-risk' females will be informative.[191] Within

this study, in the 10 women analysed, 8 were found to heterozygous, although in the case of unrelated women they were all informative.

The Taq I polymorphism has proved to be a useful marker in RFLP studies in haemophilia A but there are potential problems in the interpretation of Southern blots when supposedly constant bands may migrate with polymorphic bands obscuring important data. Experience with the interpretation of this polymorphism is essential before its use in carrier detection studies and prenatal diagnosis.

In women in whom carrier detection is only possible using linked polymorphic markers, a combined approach using the results from phenotypic analysis may increase the accuracy of diagnosis.[350] Finally, in potential carriers in whom genotypic analysis is unhelpful, it may still be possible to establish carrier status from phenotypic analysis. Therefore, even with RFLP analysis, the data from phenotypic analysis forms an essential part of carrier detection.

A number of studies have shown that carrier detection in haemophilia B can be readily performed by DNA analysis using intragenic polymorphisms,[137-146,149] and many of these RFLP's can now be rapidly analysed by PCR.[142,143,146,147] Despite significant linkage disequilibrium between closely positioned intragenic polymorphisms, it is estimated that RFLP analysis can provide a diagnosis in 89% of 'at-risk' women.[144] Unfortunately, the factor IX RFLP's show marked ethnic variation (See Chapter 1) which limits their usefulness in some populations.

In this Thesis only three intragenic polymorphisms were analysed - Taq I, Xmn I and Dde I and all women studied were heterozygous for one or more. The observed heterozygosity frequencies are based on a small sample and as a result have large sampling errors. Previously published studies show that 45% of women will be

heterozygous for the Taq I polymorphism,[120] 41% for the Xmn I polymorphism[135] and 37% for the Dde I polymorphism.[135]

Unfortunately the Taq I and Xmn I polymorphisms lie within 4.5kb of each other and show strong linkage disequilibrium and the additional data obtained from analysing both is small although sometimes useful.[352] The Dde I polymorphism is also situated close to the Taq I and Xmn I sites[135] and has been shown to be in linkage disequilibrium with both. However, in spite of this linkage disequilibrium, approximately 16% of women, homozygous for the Taq I/Xmn I polymorphisms will be informative for the Dde I polymorphism.[135]

Within this study, analysing the Xmn I polymorphism yielded no additional information over that derived from the Taq I polymorphism. Only a single family in whom the Taq I/Xmn I polymorphisms were non-informative, was analysed for the Dde I polymorphism and both 'at-risk' females were found to be heterozygous. Therefore, RFLP analysis by Southern blotting identified informative polymorphisms in all the 'at-risk' women.

Haplotype frequencies were not calculated for the three RFLP's used in this study as the number of unrelated individuals in whom data was available was small. Previous studies[135] have shown that the 11.5/1.8kb and 6.5/1.3kb haplotypes represent over 90% of chromosomes analysed. Analysis of linkage of the Dde I polymorphism to the Xmn I polymorphism showed that the rare 1.75kb Dde I allele was always associated with the frequently occurring 11.5kb Xmn I allele and that the 6.5/1.75kb haplotype was not observed although it was predicted to account for almost 5% of haplotypes - a finding consistent with linkage disequilibrium between the two polymorphic sites.

A number of potential problems with RFLP analysis are recognised which can limit their usefulness in carrier detection studies:

1. Correct paternity: when a polymorphism is used to track the inheritance of a mutation, correct identification of the biologic father is essential for test accuracy. False assumptions of paternity can result in identification of the wrong polymorphism as being associated with the mutant gene and therefore, in a mistake in both carrier detection and possibly prenatal diagnosis. As the number of family members necessary for establishing a linkage phase increases, the greater the chances of error due to false paternity. For X-linked diseases false paternity in a fetus or male offspring is not a source of error in diagnosis but false paternity can cause an error in the determination of carrier status and the establishment of linkage phases in females. Although difficult to discuss with families seeking genetic counselling, they must be made aware of the problems that false paternity can cause. Paternity testing should be ascertained whenever information from paternal RFLP's is used and in addition when a recombination event is suspected. In these situations, genetic fingerprinting using minisatellite probes may prove to be extremely useful.

2. Missing 'key' family members: in some families and in particular those in which there is only a single isolated affected male, analysis of all family members may be essential in establishing the origin of the mutant gene and, therefore, in identifying which females are at risk of being carriers.

3. Homozygosity for all polymorphisms: the X chromosome appears to be less polymorphic than the autosomes, and a number of individuals will, therefore, be homozygous for all the available polymorphisms. In these women, the only possible hope for carrier detection is identification of the underlying genetic mutation.

4. Linkage disequilibrium.

5. Recombination: the risk of a recombination occurring in genetic analyses is directly related to the distance between the locus of the polymorphism and the gene of interest and increases as the distance between the two increases. These risks can be incorporated

into Bayesian analysis (See Chapter 1).

In this study three recombinations were found to have occurred between the DXS15 (Bgl II) and Bcl I loci. In one of these families the recombination occurred between the Bgl I/Bcl I loci and the DXS15 locus. Harper in his original paper[190] estimated the recombination rate between the FVIII gene and the DXS15 locus would be less than 9%. Further studies[192,334,353,354] have estimated this risk at between 3-5%. Within this study, a recombination rate of 7%, slightly higher than previously published figures, was found between the DXS15 and Bcl I loci. No recombinations were found between the Xba I and DXS15 loci.

Although a number of recombinations have been reported between the FVIII gene and the DXS52 (ST14) loci[334,355,356] no definite cross-overs were found within this study although in one family (Pedigree No. 39) the limited genotypic data suggested that this was a possibility although false paternity is more likely. The number of women in whom the DXS52 locus was studied is small and the data is, therefore, limited. However, a single recombination between the DXS15 and DXS52 loci was identified in a family with X-linked adrenoleukodystrophy (Pedigree No. C1: Appendix 3) although again accurate paternity was not established.

Despite these problems RFLP analysis has dramatically improved carrier detection in haemophilia A and B increasing both the availability and the accuracy of diagnosis. In this study, combined phenotypic/genotypic assessment of carrier status was possible in 48/80 (60%) of potential carriers and informative polymorphisms were identified in 38/40 (95%) obligate carriers.

One of the principal objectives of carrier detection studies in haemophilia is to clarify the

carrier status of potential carriers. Whilst phenotypic analysis is based on probabilities, genotypic analysis using intragenic RFLP's allows a definite decision to be made increasing the accuracy of genetic counselling and allows individuals to make decisions about whether or not to have antenatal diagnosis and terminate an affected pregnancy. By combining the results from phenotypic and genotypic studies and using Bayesian analysis a final probability of carriership can be established for all 'at-risk' women.

Within this study 80 potential carriers of haemophilia A were identified, 32 were from families in which there was a history of haemophilia for more than a single generation or in whom the birth of a second affected child established the mother as an obligate carrier and the remainder were from families in whom there was no previous history of haemophilia.

From the results of coagulation phenotype analysis it was possible to assign a potential carrier to one of two groups - normal coagulation phenotype or abnormal coagulation phenotype. (This assignment is based upon an arbitrarily assigned 'cut-off' value and is, therefore, open to criticism.) In a small group of women no phenotypic data was available, although genotypic analysis was still possible.

Following RFLP studies, potential carriers could be reclassified into definite carriers, normal women or if the results were inconclusive - they remained potential carriers. Of the 80 potential carriers studied, it was possible to definitively establish the carrier status in 48 women. Of these 48 women, 17 were shown to be carriers and of these 12 had an abnormal coagulation phenotype. The remaining 31 women were shown to be normal and of these 25 had a normal coagulation phenotype. Within the first group, genotypic analysis reclassified 2 women with normal phenotypes as carriers, whilst in the second group, subsequent analyses reclassified 4 women as normal. In 32 women, genotypic analysis could not establish their carrier status either because they were homozygous for

the polymorphisms analysed or key family members were missing.

From the 4 families with haemophilia B, 8 potential carriers were identified. Phenotypic/genotypic analysis allowed the carrier status of 2 to be established as carriers but in the remainder analyses were inconclusive and these women remain potential carriers. In this situation, the identification of a kindred specific defect should allow clarification of the carrier status of all 'at-risk' women.

Although fatal without treatment, the frequency of haemophilia is maintained within a population indicating that new mutations are continually arising. Haldane in 1935 was the first person to examine the possibility of a sex difference in mutation rates during gematogenesis.[357] His formulation suggested that for an X-linked lethal disorder, if the mutation rates are equal in both sexes, then the probability of an isolated case being the result of a new mutation is:

$$m = \frac{(1-f)u}{2u+v}$$

where f = biological fitness, μ = mutation rate in females and v = the mutation rate in males. If the biological fitness equals zero ie. no patients reproduce and the mutation rate in male and female gametes is the same then the probability of an isolated case being the result of a new mutation is:

$$= \frac{1-0}{(2\times 1)+1}$$

$$= \frac{1}{3}$$

However, in haemophilia the mutation rate in male gametes is estimated to be approximately 10X that in female gametes and the biological fitness is estimated at

between 0.3 and 0.7.[324] Using these variables in the formula the proportion of isolated cases which are due to new mutations lies between 2.5 (f=0.3) and 5.8% (f=0.7). This is significantly less than the figure of 30% which has been suggested by Bennett and Ratnoff [282] but in keeping with the findings of Biggs and Rizza.[358] This has important implications for genetic counselling as it suggests that the vast majority of women with an isolated affected haemophilic child are carriers and, therefore, at risk of having a further affected child.

Within a kindred with a single isolated affected male it may be possible to identify the origin of the haemophilic gene by a combination of genotypic and phenotypic analysis greatly facilitating carrier detection.

Within this study there were 18 families comprising 48 potential carriers in whom there was a single isolated affected male. Phenotypic analysis identified 17 women as having an VIII:C/vWF:Ag ratio consistent with that of a carrier, 29 as normal and in 2 no coagulation data was available. Combined phenotypic/genotypic analysis allowed 24/48 (50%) of these potential carriers to be reclassified as either normal or carriers. In the remaining 24 women, carriership could not be established either because insufficient pedigree data was available or the polymorphisms studied were non-informative.

In sporadic haemophilia A, establishing carrier status usually depends upon demonstrating that an 'at-risk' female has not inherited the potentially abnormal haplotype ie. a diagnosis of exclusion. A number of studies have evaluated the role of RFLP analysis in sporadic haemophilia A. Grover in 1987[359] using two intragenic (Bcl I and Bgl I) and two linked RFLP's (Taq I, Bgl II) assessed the carrier status of 30 women from 5 families with an isolated affected male. In 3 families they were able to assign a low risk to 10 women based on genotypic analysis, in a further 2 families

genotypic analysis suggested the mutation originated in either the maternal grandmother's or maternal grandfather's germ cells thereby limiting the risk to their descendants. In the remaining family a large gene deletion was identified enabling carrier detection by densitometric analysis. In the same year Bernardi[360] using three intragenic and two linked RFLP's studied 17 families with isolated affected males. In 8 of these families they were able to show that the mutated genes originated with one of the maternal grandparents; in six families of these families the mutations derived from the maternal grandfather whilst in the remaining two families from the maternal grandmother. This data suggested a higher mutation rate in male rather than female germ cells and may be explained by the many more cell divisions which occur in the maturation of spermatozoa (10-20X) than in oocytes.[361]

Lillicrap in 1988[350] studied the benefits of combined RFLP and coagulation testing in sporadic haemophilia A and was able to establish 23 women as normal but 27 remained at risk after RFLP analysis. Of these 27, 14 had coagulation test results which indicated a high probability of carriership. They concluded that an optimal service for haemophilia A carrier detection must offer coagulation testing in addition to DNA marker analysis. Antonarakis and Kazazian[362] were able to characterise the mutation in 13 apparently spontaneous cases of haemophilia A. In 11/13 cases they could identify the origin of the mutation; 5/11 occurred in the maternal grandfathers and the remaining 6 in either the mother or grandmother.

Within this study, the origin of the mutation could be predicted in 8 of the 18 families by RFLP analysis. In 2 families the mutation appeared to be unique to the affected haemophiliac. In 3 families it appeared to have originated with the maternal grandfather and in each case his daughters had abnormal coagulation phenotypes. In a further 2 families although the grandfather could not be definitely established as the origin of the mutations the genotypic data favoured him as the source. In the remaining family the

mutation could be tracked as far as the maternal grandmother implicating her as a possible carrier and this was substantiated by an abnormal coagulation phenotype. These findings strengthen the general impression that the maternal grandfather appears to be the source of the haemophilic mutation in spontaneous haemophilia A more frequently than the grandmother or mother.

Mutations in families with sporadic haemophilia A are presumed to arise at the level of the germ line and the majority probably occur during the development of the germ cells so that a mutated gamete is unique. Consequently, the siblings of individuals with new mutations are unaffected. If, however, the mutation occurs early in embryogenesis before differentiation of the germ line, then the individual with the mutation will be a mosaic and if a gonadal mosaic may have several types of germ cells. In some cases of mosaicism, in which a high proportion of the somatic cells contain the mutation eg. leucocytes, this may be detectable by Southern Blotting - but if the defect is not readily detectable then this is not possible. As a result, the recurrence risk in some families with apparent sporadic disease will be higher than expected. Although mosaicism in haemophilia A has been reported[363] it is unclear how often it accounts for spontaneous disease and further work is required to clarify this area.

Tracking the haemophilic haplotype through various generations of a pedigree emphasises the importance of extended pedigree data in families in whom the disease appears to be spontaneous and in particular genotypic analysis of parents, grandparents and any siblings. It is possible that by extending the family pedigree data, the number of families in which this type of analysis could be performed could be increased. Tracking the mutation by RFLP analysis is extremely important in families with sporadic haemophilia A as it allows more accurate carrier detection studies and increases the information which can be given to 'at-risk' females during genetic counselling. However, such studies require extensive pedigree, phenotypic and genotypic data. In

sporadic haemophilic families in which genotypic analysis fails to establish the carrier status of potential carriers, phenotypic analysis may still allow their risk of carriership to be predicted.

Unfortunately, in the 2 families with sporadic haemophilia B, only limited data was available and the origin of the mutation could not be identified. With the identification of kindred specific mutations in haemophilia B the origin of the mutation can be accurately predicted and accurate genetic counselling given.

The logical conclusion of carrier testing in haemophilia A and B and the identification of informative polymorphisms is to be able to accurately identify carriers and to offer antenatal diagnosis if requested. Conventional techniques for prenatal diagnosis in 'at-risk' women involves fetal sexing followed by blood sampling at a relatively late stage in pregnancy. Genotypic analysis which can be performed on chorionic villus samples (CVS), therefore allows the early and rapid detection of affected fetus and by using PCR in previously tested informative families results can be available within 24 hours. Within this study the availability of carrier detection using genotypic data, phenotypic data or a combination of the two was assessed.

In haemophilia A prenatal diagnosis could be offered to all 'at-risk' women studied using either informative intragenic polymorphisms (33/89), a combination of extragenic polymorphisms and fetal blood sampling (39/89) or by fetal blood sampling alone (17/89). In women in whom carrier detection studies fails to establish their carrier status, fetal sexing and genotypic analysis (if informative) can identify 'at-risk' pregnancies but confirmation of an affected male by fetal blood sampling will be required. In general, fetal blood sampling usually involves the measurement of VIII:C although the measurement of VIII:Ag has been shown to correlate well with VIII:C in CRM^-

families.[109]

The value of VIII:Ag measurements in carrier detection has not been assessed in this Thesis - such assays were not available commercially and previous studies have shown no improvements over measurements of VIII:C.[296] However, VIII:Ag is more stable than VIII:C it can be measured in serum and may, therefore have a role in prenatal diagnosis based on fetal blood sampling and in assessing FVIII levels from geographically distant centres when measurements of VIII:C are not possible.

In an analogous situation to that of haemophilia A, antenatal diagnosis can be offered to all the women in the haemophilia B families. All of the obligate carriers and the two potential carriers shown by combined phenotypic/genotypic analysis to be definite carriers, were informative for one or more intragenic polymorphisms. However, for the remaining potential carriers although prenatal diagnosis is possible using intragenic polymorphisms and chorionic villus sampling, carriership in the mothers has not been definitively established necessitating confirmation of an affected pregnancy by fetal blood sampling. With the development of techniques for rapidly identifying kindred specific defects, genotypic analysis of carriers will be readily available allowing both accurate carrier detection and prenatal diagnosis.

During the course of this study into carrier detection in haemophilia, two particularly interesting families were identified. In the first of these - Pedigree No. 42 (Appendix 1), although there was no history of haemophilia A the coagulation results were consistent with a diagnosis of a heterozygous female ie. a haemophilia A carrier. However, an unusual form of von Willebrand's disease affecting the VIII:C binding site could not be excluded.[364] It is obviously vital to diagnose these individuals with variant vWD as both the treatment and genetic counselling are entirely different to that of classical haemophilia A. FVIII binding studies to clarify whether this variant vWF is present in the family reported in this Thesis are currently in progress.

In the second family - Pedigree No. B5 (Appendix 2), although there was no family history of haemophilia B, a single female was identified as having low levels of both IX:C and IX:Ag in association with both an abnormal karyotype and a point mutation within one FIX gene.

The commonest explanation for the finding of low factor VIII or IX levels in a woman is extreme Lyonisation in a heterozygote. However, additional possibilities include misdiagnosed von Willebrand's disease (low VIII:C); genetic abnormality in a phenotypic female with one X-chromosome carrying an abnormal FVIII or FIX gene eg. testicular feminisation (46,XY), Turner's Syndrome (XO) or Turner's mosaic (XX/XO) and genetic abnormality in a phenotypic female (deletions, inversions, translocations involving one of the two X-chromosomes with extreme Lyonisation of the other.

In the female identified within the study, the cause of the low FIX levels could not be established as she has both an abnormal karyotype and a mutation within her FIX gene. Interestingly, she has a daughter in whom both FIX assays and karyotypic analysis are normal although DNA was not available for molecular analysis.

A single family with X-linked adrenoleukodystrophy (ALD) was analysed during this study. Previous work has shown that the linked DXS52 (ST14) marker is useful in carrier detection studies in ALD and appears to be associated with a low risk of recombination. Analysis of this marker reduced the risk to one potential carrier of carriership and in addition, identified a recombination between the DXS15 and DXS52 loci. Such a recombination is likely to be a rare event as these two markers are physically close to one another on the X-chromosome and non-paternity must, therefore, be excluded.

The Future of Carrier Testing In Haemophilia.

Improvements in coagulation testing and development of genotypic analysis using the study of RFLP's has radically improved the detection of haemophilia carriers. Whilst progress in RFLP analysis continues to be made eg. direct detection of RFLP's by PCR, the use of variable number tandem repeats (VNTR's), the future of carrier testing must lie with the molecular characterisation of the mutant gene in specific families. Identification of the mutation in affected individuals will allow rapid carrier detection in 'at-risk' females, allow for early, accurate antenatal diagnosis and in addition, will increase our understanding of the molecular basis of both haemophilia A and B. This approach will be of benefit to all families but in particular those with sporadic disease in whom RFLP analysis is unhelpful. However, such an approach is likely to be demanding in terms of technical expertise, equipment and the interpretation of the results and may indicate a need for the establishment of a small number of laboratories where families may be referred for further investigation.

The ultimate hope for haemophilia is that some form of gene therapy may provide a 'cure'. Meanwhile, advances in *in vitro* fertilisation may allow the selection of non-haemophilic males as an alternative to antenatal diagnosis and abortion.

Bibliography.

1. Marder VJ, Mannucci PM, Firkin BG, Hoyer LW, Meyer D. Standard Nomenclature for Factor VIII and von Willebrand Factor: A recommendation by the International Committee on Thrombosis and Haemostasis. Thrombosis and Haemostasis 1985;54(4):871-872.

2. Chen TI, Tsai C. The mechanism of haemostasis in peripheral vessels. Journal of Physiology 1948;107:280-288.

3. Baumgartner HR. Morphometric quantitation of adherence of platelets to an artificial surface and components of connective tissue. Thrombosis et Diathesis Haemorrhagica Supplement 1974;60:39-49.

4. Tschopp TB, Baumgartner HR. Physiological experiments in haemostasis and thrombosis. British Journal of Haematology 1975;31 (Suppl):221-229.

5. Niewiarowski S, Bankowski E, Rogowicka I. Studies on the absorption and activation of the Hageman factor (Factor XII) by collagen and elastin. Thrombosis et Diathesis Haemorrhagica 1965:14:387-400.

6. Tschopp TB, Weiss HJ, Baumgartner HR. Decreased adhesion of platelets to subendothelium in von Willebrand's Disease. Journal of Laboratory and Clinical Medicine 1974;83:296-300.

7. Weiss HJ, Baumgartner HR, Tschopp TB, Turitto VT, Cohen D. Correction by factor VIII of the impaired platelet adhesion to subendothelium in von Willebrand disease. Blood 1978;51:267-269.

8. Born GVR. Observations on the change in shape of blood platelets brought about by adenosine diphosphate. Journal of Physiology 1970;209:487-511.

9. Hovig T. Megakaryocyte and platelet morphology. In: Caen JP (ed.) Clinical Haematology, Baillière Tindall, London 1989:503-541.

10. O'Brien DP. The molecular biology and biochemistry of tissue factor. In: Tuddenham EGD (ed.) Clinical Haematology, Baillière Tindall, London. 1989;2(4):801-820.

11. Davie EW, Ratnoff OD. Waterfall Sequence for Intrinsic Blood Clotting. Science 1964;145:1310-1312.

12. Macfarlane RG. An Enzyme Cascade in the Blood Clotting Mechanism and its Function as a Biochemical Amplifier. Nature 1964;202:498-499.

13. Lämmle B, Griffin JH. Formation of the Fibrin Clot: the Balance of Procoagulant and Inhibitory Factors. In: Ruggeri ZM (ed.) Clinics in Haematology, Baillière Tindall, London 1985;2:281-342.

14. Jackson CM, Nemerson Y. Blood Coagulation. Annual Review of Biochemistry 1980;49:765-811.

15. Rodeghiero F, Castaman G, Dini E. Epidemiological investigation of the prevalence of von Willebrand's disease. Blood 1987;69:454-459.

16. McKee PA. Haemostasis and disorders of blood coagulation. In: Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS (eds.) The Metabolic Basis of Inherited Disease 1983, McGraw-Hill, New York. 5th. edition, pp. 1531-1460.

17. Rosner F. Hemophilia in the Talmud and Rabbinic writings. Annals of Internal Medicine 1969;70:833-837.

18. Otto JC. An account of an haemorrhagic disposition existing in certain families. Medical Repository 1803;6:1-4.

19. Hopff F. Ueber die Haemophilie oder die erbliche Anlage zu tödtlichen Blutungen. Inaug. Diss. Wurzburg 1828.

20. Bulloch W, Fildes, P. Haemophilia in Treasury of Human Inheritance. Dulau & Co., London. 1911, Parts V-V1.

21. Addis T. The pathogenesis of hereditary haemophilia. Journal of Pathology and Bacteriology 1911;15:247-452.

22. Patek AJ, Taylor FHL. Hemophilia II. Some properties of a substance obtained from normal human plasma effective in accelerating the coagulation of hemophilic blood. Journal of Clinical Investigation 1937;16:113-124.

23. Pavlovsky A. Contribution to the pathogenesis of hemophilia. Blood 1947;2:185-191.

24. Schulman I, Smith CH. Hemorrhagic disease in an infant due to deficiency of a previously undescribed clotting factor. Blood 1952;7:794-807.

25. Aggeler PM, White SG, Glendening MB, Page EW, Leake TB, Bates G. Plasma Thromboplastin Component (PTC) Deficiency: A new disease resembling hemophilia. Proceedings of the Society for Experimental Biology and Medicine 1952;79:692-694.

26. Biggs R, Douglas AS, Macfarlane RG, Dacie JV, Pitney WR, Merskey C, O'Brien JR. Christmas Disease a condition previously mistaken for haemophilia. British Medical Journal 1952;ii:1378-1382.

27. Wright IS. The nomenclature of blood clotting factors. Thrombosis et Diathesis Haemorrhagica 1962;7:381-382.

28. Foster PA, Zimmerman TS. Factor VIII Structure and Function. Blood Reviews 1989;3:180-191.

29. Tuddenham EGD. Factor VIII and Haemophilia A. In: Tuddenham EGD (ed.) Clinical Haematology. Baillière Tindall, London. 1989;2(4):849-877.

30. Griffit MJ, Reisner HM, Lundblad RL, Roberts HR. Measurement of human factor IXa activity in an isolated factor X activation system. Thrombosis Research 1982;27:289-301.

31. Andersson L-O, Brown JE. Interaction of factor VIII-von Willebrand Factor with phospholipid vesicles. Biochemical Journal 1981;200:161-167.

32. Koedam JA, Meijers JCM, Sixma JJ, Bouma BN. Von Willebrand factor protects factor VIII from inactivation by activated Protein C and Protein S. Thrombosis and Haemostasis 1987;58:223a.

33. Hamer RJ, Koedam JA, Beeser-Visser NH, Sixma JJ. The effect of thrombin on the complex between factor VIII and von Willebrand factor. European Journal of Biochemistry 1987;167:253-259.

34. Preston AE, Barr A. The plasma level of factor VIII in the normal population. British Journal of Haematology 1964;10:238-245.

35. Hjort PF, Egberg O, Mikkelsen S. Turnover of prothrombin, factor VII and factor IX in a patient with hemophilia A. Scandinavian Journal of Clinical and Laboratory Investigation 1961;13:668-672.

36. von Willebrand EA. Hereditar pseudohamofili. Finsk Lakaresallsk Handl 1926;68:87-112.

37. von Willebrand EA, Jurgens R. Über ein neues verebbares Blutungubel: die konstitutionelle thrombopathie. Dtsch Arch Klin Med 1933;175:453-483.

38. Alexander B, Goldstein R. Dual hemostatic defect in pseudohemophilia. Journal of Clinical Investigation 1953;32:551.

39. Larrieu MJ, Soulier JP. Deficit en facteur antihemophilique A chez une fille associé a un trouble du saignment. Revue D'Hématologie 1953;8:361-370.

40. Quick AJ, Hussey CV. Hemophilic condition in the female. Journal of Laboratory and Clinical Medicine 1953;42:929-930.

41. Holmberg L, Nilsson IM. von Willebrand Disease. In: Ruggeri ZM (ed.) Clinics in Haematology. W.B. Saunders Co., London. 1985;14(2):461-488.

42. Nilsson IM, Blombäck M, von Francken I. On an inherited autosomal hemorrhagic diathesis with antihemophilic globulin (AHG) deficiency and prolonged bleeding time. Acta Medica Scandinavica 1957;159:35-57.

43. Borchgrevink CF. Platelet adhesion in vivo in patients with bleeding disorders. Acta Medica Scandinavica 1961;170:231-243.

44. Zimmerman T, Ratnoff OD, Powell AE. Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease, with observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and an acquired circulating anticoagulant against antihemophilic factor. Journal of Clinical Investigation 1971;50:244-254.

45. Howard MA, Firkin BG. Ristocetin: A new tool in the investigation of platelet aggregation. Thrombosis et Diathesis Haemorrhagica 1971;26:362-369.

46. Weiss HJ, Rogers J, Brand H. Defective ristocetin-Induced Platelet Aggregation in von Willebrand's Disease and its Correction by Factor VIII. Journal of Clinical Investigation 1973;52:2697-2707.

47. Thelin GM, Wagner RH. Sedimentation of plasma antihemophilic factor. Archives of Biochemistry and Biophysics 1961;95:70-76.

48. Weiss HJ, Hoyer LW. Von Willebrand factor: dissociation from antihemophilic factor coagulant activity. Science 1973;182:1149-1151.

49. Owen WG, Wagner RH. Antihemophilic factor: separation of an active fragment following dissociation by salts or detergents. Thrombosis et Diathesis Haemorrhagica 1972;27:502-515.

50. Cooper HA, Griggs TR, Wagner RH. Factor VIII recombination after dissociation by $CaCl_2$. Proceedings of the National Academy of Sciences USA 1973;70:2326-2329.

51. Ginsburg D, Handin RI, Bonthron DT, Donlon TA, Bruns GAP, Latt SA, Orkin SH. Human von Willebrand Factor (vWF): Isolation of Complementary DNA (cDNA) Clones and Chromosomal Localization. Science 1985;228:1401-1406.

52. Lynch DC, Zimmerman TS, Collins CJ. Molecular cloning of cDNA for human von Willebrand factor: authentication by a new method. Cell 1985;41:49-56.

53. Sadler JE, Shelton-Inloes BB, Sorace JM, Harlan JM, Titani K, Davie EW. Cloning and characterization of two cDNA's coding for human von Willebrand factor. Proceedings of the National Academy of Sciences USA 1985;82:6394-6398.

54. Verweij CL, De Vries CJM, Distel B, Van Zonneveld AJ, Van Kessel AG, Mourik JA, Pannekoek H. Construction of cDNA coding for human von Willebrand factor using antibody probes for colony-screening and mapping of the chromosomal gene. Nucleic Acids Research 1985;13(13):4699-4717.

55. Shelton-Inloes BB, Titani K, Sadler JE. cDNA Sequences for Human Von Willebrand Factor Reveal Five Types of Repeated Domains and Five Possible Protein Sequence Polymorphisms. Biochemistry 1986;25:3164-3171.

56. Mancuso DJ, Tuley EA, Westfield LA, Worrall NK, Shelton-Inloes BB, Sorace JM, Alevy YG, Sadler JE. Comparison of the human von Willebrand factor gene and pseudogene structures. Circulation 1988;78(Suppl. II):506.

57. Verweij CL, Diergaarde PJ, Hart M, Pannekoek H. Full length von Willebrand factor (vWF) cDNA encodes a highly repetitive protein considerably larger than the mature vWF subunit. EMBO Journal 1986;5:1839-1847.

58. Bonthron DT, Handin RI, Kaufman RJ, Wasley LC, Orr EC, Mitsock LM, Ewenstein B, Loscalzo J, Ginsburg D, Orkin SH. Structure of the pre-pro-von Willebrand factor and its expression in heterologous cells. Nature 1986;324:270-273.

59. Titani K, Kumar S, Takio K, Ericsson LH, Wade RD, Ashida K, Walsh K, Chopek MW, Sadler JE, Fujikawa K. Amino Acid Sequence of Human von Willebrand Factor. Biochemistry 1986;25:3171-3184.

60. Girma J-P, Meyer D, Verweij CL, Pannekoek H, Sixma JJ. Structure-Function Relationship of Human Von Willebrand factor. Blood 1987; 70(3):605-611.

61. Colombatti A, Bonaldo P. The Superfamily of Proteins With von Willebrand Factor Type-A-Like Domains: One Theme Common to Components of Extracellular Matrix, Hemostasis, Cellular Adhesion and Defence Mechanisms. Blood 1991;77(11):2305-2315.

62. Fowler WE, Fretto LJ, Hamilton KK, Erickson HP, McKee PA. Substructure of human von Willebrand factor. Journal of Clinical Investigation 1985;76:1491-1500.

63. Fay PJ, Kawai Y, Wagner DD, Ginsburg D, Bonthron D, Ohlsson-Wilhelm BM, Chavin SI, Abraham GN, Handin RI, Orkin SH, Montgomery RR, Marder VJ. Propolypeptide of von Willebrand factor circulates in blood and is identical to von Willebrand antigen II. Science 1986;232:995-998.

64. McCarroll DR, Levin EG, Montgomery RR. Endothelial cell synthesis of von Willebrand antigen II, von Willebrand factor and von Willebrand factor/von Willebrand antigen II complex. Journal of Clinical Investigation 1985;75:1089-1095.

65. Montgomery RR, Zimmerman TS. von Willebrand's disease antigen II: A new plasma and platelet antigen deficient in severe von Willebrand's disease. Journal of Clinical Investigation 1978;61:1498-1507.

66. Wise RJ, Pittman DD, Hamdin RI, Kaufman RJ, Orkin SH., The propeptide of von Willebrand factor independently mediates the assembly of von Willebrand multimers. Cell 1988; 52:229-236.

67. Verweij CL, Hart M, Pannekoek H. Expression of variant von Willebrand factor (vWF) cDNA in heterologous cells: requirement of the pro-polypeptide in vWF multimer formation. EMBO J 1987;6:2885-2890.

68. Loesberg C, Gonsalves MD, Zandbergen J, Willems C, Van Aken WG, Stel HV, van Mourik JA, De Groot PG. The effect of calcium on the secretion of factor VIII-related antigen by cultured human endothelial cells. Biochemica et Biophysica Acta 1983;763:160-168.

69. Sporn LA, Marder VJ, Wagner DD. Inducible secretion of large, biologically potent, von Willebrand factor multimers. Cell 1986;46:185-190.

70. Zucker MB, Broekman MJ, Kaplan KL. Factor VIII-related antigen in human blood platelets. Localization and release by thrombin and collagen. Journal of Laboratory and Clinical Medicine 1979;94:675-682.

71. Ruggeri ZM, Zimmerman TS. The complex multimeric composition of factor VIII/von Willebrand factor. Blood 1981;57:1140-1143.

72. Baruch D, Bahnak B, Girma J-P, Meyer D. Von Willebrand factor and platelet function. In: Caen JP (ed.) Clinical Haematology, Baillière Tindall, London. 1989;2(3):627-672.

73. Baumgartner HR, Tschopp TB, Meyer D, Turitto VT, Weiss HJ. Von Willebrand factor in platelet interaction with subendothelium. Thrombosis and Haemostasis 1981;46:157.

74. Jenkins CSP, Clemetson KJ, Lusher EF. Studies on the mechanism of ristocetin-induced platelet agglutination: binding of ristocetin to platelets. Journal of Laboratory and Clinical Medicine 1979;93:220-231.

75. Moake JL, Olson JD, Troll JH, Tng SS, Funicella T, Peterson DM. Binding of radioiodinated human von Willebrand factor to Bernard-Soulier thrombasthenic and von Willebrand's disease platelets. Thrombosis Research 1980;19:21-27.

76. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. Journal of Clinical Investigation 1983;72:325-338.

77. Fujimoto T, Hawiger J. Adenosine diphosphate induces binding of von Willebrand factor to human platelets. Nature 1982;297:154-156.

78. Fujimoto T, Ohara S, Hawiger J. Thrombin-induced exposure and prostacyclin inhibition of the receptor for factor VIII/von Willebrand factor on human platelets. Journal of Clinical Investigation 1982;69:1212-1222.

79. Ruggeri ZM, Bader R, De Marco L. Glanzmann thrombasthenia: deficient binding of von Willebrand factor to thrombin-stimulated platelets. Proceedings of the National Academy of Sciences USA 1982;79:6038-6041.

80. Ruggeri ZM, Bader R, Pareti FI, Mannucci L, Zimmerman TS. High affinity interaction of platelet von Willebrand factor with distinct platelet membrane sites. Clinical Research 1983;31:322a.

81. Santoro SA, Cowan JF. Adsorption of von Willebrand factor by fibrillar collagen. Implications concerning the adhesion of platelets to collagen. Collagen and Related Research. 1982;2:31-43.

82. Morton LF, Griffin B, Pepper DS, Barnes MJ. The interaction between collagens and factor VIII/von Willebrand factor: investigation of the structural requirements for interaction. Thrombosis Research 1983;32:545-556.

83. De Groot PG, Ottenhof-Rovers M, van Mourik JA, Sixma JJ. Evidence that the primary binding site of von Willebrand factor that mediates platelet adhesion on subendothelium is not collagen. Journal of Clinical Investigation 1979;82:65-73.

84. Weiss HJ, Sussman II, Hoyer LW. Stabilization of factor VIII in plasma by the von Willebrand factor. Journal of Clinical Investigation 1977;60:390-404.

85. Foster PA, Fulcher CA, Marti T, Titani K, Zimmerman TS. A major factor VIII binding domain resides within the amino-terminal 272 amino acid residues of von Willebrand factor. Journal of Biological Chemistry 1987;262:8443-8446,

86. Takahashi Y, Kalafatis M, Girma J-P, Sewerin K, Andersson L-O, Meyer D. Localization of a factor VIII binding domain on a 34 kiloDalton fragment of the N-terminal portion of von Willebrand factor. Blood 1987;70:1679-1682.

87. Wright AE. On a method of determining the condition of blood coagulability for clinical and experimental purposes, and on the effect of the administration of calcium salts in haemophilia and actual or threatened haemorrhage. British Medical Journal 1893;2:223-225.

88. Addis T. The pathogenesis of hereditary haemophilia. Journal of Pathology and Bacteriology 1911;15:427-452.

89. Brinkhous KM. A study of the clotting time in hemophilia: the delayed formation of thrombin. American Journal of Medical Sciences 1939;198:509-516.

90. Merskey C. Haemophilia associated with a normal coagulation time. British Medical Journal 1951;i:906-912.

91. Biggs R, Douglas AS. The thromboplastin generation test. Journal of Clinical Pathology 1953;6:23-29.

92. Kirkwood TBL, Barrowcliffe TW. Discrepancy between One-stage and Two-stage Assay of Factor VIII:C. British Journal of Haematology 1978;40:333-338.

93. Brandt JT, Tripless DA, Musgrave K, Arkin C, Bovill EG, Lucas FV, Rock WA. Factor VIII Assays: Assessment of Variables. Archives of Pathology and Laboratory Medicine 1988;112:7-12.

94. Kirkwood TBL, Rizza CR, Snape TJ, Rhymes IL, Austen DEG. Identification of Sources of Inter-Laboratory Variation in Factor VIII Assay. British Journal of Haematology 1977;37:59-568.

95. Christensen RL, Triplett DA. Factor Assay (VIII and IX) results in the College of American Pathologists Survey Program (1980-1982). American Journal of Clinical Pathology 1983;80:633-642.

96. Howard MA, Sawers RJ, Firkin BG. Ristocetin: A means of differentiating von Willebrand's Disease Into Two Groups Blood. Blood 1973;41(5):687-690.

97. Weiss HJ, Rogers J, Brand H. Defective ristocetin-Induced Platelet Aggregation in von Willebrand's Disease and its Correction by Factor VIII. The Journal of Clinical Investigation 1973;52:2697-2707.

98. Macfarlane DE, Stibbe J, Kirby EP, Zucker MB, Grant RA, McPherson J. A method for assaying von Willebrand factor (ristocetin cofactor). Thrombosis et Diathesis Haemorrhagica 1975;34:306-308.

99. Zuzel M, Nilsson IM, Aberg M. A method for measuring plasma ristocetin cofactor activity. Normal distribution and stability during storage. Thrombosis Research 1978;12:745-754.

100. Chand S, McCraw A, Hutton R, Tuddenham EGD, Goodall AH. A Two-Site, Monoclonal Antibody-Based Immunoassay for von Willebrand Factor - Demonstration that vWF Function Resides in a Conformational Epitope. Thrombosis and Haemostasis 1986;55(3):318-324.

101. Zimmerman TS, Roberts T, Ruggeri ZM. Factor VIII-related Antigen: Characterization by electrophoretic Techniques. In: Bloom AL (ed.) The Hemophilias. Churchill Livingstone, London, pp.81-91.

102. Ruggeri ZM, Zimmerman TS. Variant von Willebrand's disease: characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. Journal of Clinical Investigation 1980;65:1318-1325.

103. Ciavarella G, Ciavarella N, Antonecchi S, De Mattia D, Ranieri P, Dent J, Zimmerman TS, Ruggeri ZM. High-resolution analysis of von Willebrand factor multimeric composition defines a new variant of type I von Willebrand disease with aberrant structure but presence of all size multimers (type IC). Blood 1985;66:1423-1429.

104. Fantl P, Sawers RJ, Marr AG. Investigation of a hemorrhagic disease due to beta-thromboplastin deficiency complicated by a specific inhibitor of thromboplastin formation. Australasian Annals of Medicine 1956;5:163-176.

105. Roberts HR, Gross GP, Webster WP, Dejanov II, Penick GD. Acquired inhibitors of plasma factor IX: A study of their induction, properties, and neutralization. American Journal of Medical Science 1966;251:43-50.

106. Hougie C, Twomey JJ. Hemophilia B_m: A new type of factor IX deficiency. Lancet 1967;i:698.

107. McCgraw RA, Davis LM, Lundblad RL, Stafford SW, Roberts HR. Structure and Function of Factor IX: Defects in Haemophilia B. In: Ruggeri ZM (ed.) Clinics in Haematology. WB Saunders Co., London. 1989;14(2):359-383.

108. Laurell CB. Quantitative estimation of proteins by electrophoresis in agarose gels containing antibodies. Analytical Biochemistry 1966;15:45-52.

109. Peake IR, Bloom AL, Giddings JC, Ludlam CA. An immunoradiometric assay for procoagulant factor VIII antigen: results in haemophilia, von Willebrands's disease and fetal plasma and serum. British Journal of Haematology 1979;42:269-281.

110. Furlong RA, Peake IR, Bloom AL. Factor VIII clotting antigen (VIIIC:Ag) in haemophilia measured in two immunoradiometric assays (IRMA) using different antibodies and the measurement of inhibitors to procoagulant factor VIII (VIII:C) by IRMA. British Journal of Haematology 1981;48:643-650.

111. DiScipio RG, Kurachi K, Davie EW. Activation of human factor IX (Christmas factor). Journal of Clinical Investigation 1978;61:1528-1537.

112. Fukikawa K, Thompson AR, Legaz ME, Meyer RG, Davie EW. Isolation and characterization of bovine factor IX (Christmas factor). Biochemistry 1973;12:4938-4945.

113. Kurachi K, Davie EW. Isolation and characterization of a cDNA coding for human factor IX. Proceedings of the National Academy of Sciences USA 1982;79:6461-6464.

114. Choo KH, Gould KG, Rees DJG, Brownlee GG. Molecular cloning of the gene for human anti-haemophilic factor IX. Nature 1982;299:178-180.

115. Jaye MH, De La Salle H, Schamber F, Balland A, Kohli V, Findeli A, Tolstoshev P, Lecocq JP. Isolation of a human anti-haemophilic factor IX cDNA clone using a unique 52-base synthetic oligonucleotide probe deduced from the amino acid sequence of bovine factor IX. Nucleic Acids Research 1983;11(8):2325-2335.

116. Anson DS, Choo KH, Rees DJG, Giannelli F, Gould K, Huddleston JA, Brownlee GG. The gene structure of human anti-haemophilic factor IX. EMBO 1984;3(5):1053-1060.

117. Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K. Nucleotide Sequence of the Gene for Human Factor IX (Antihemophilic Factor B). Biochemistry 1985;24:3736-3750.

118. Chance PF, Dyer KA, Kurachi K, Yoshitake S, Ropers H.-H, Wieacker P, Gartler SM. Regional localization of the human factor IX gene by molecular hybridization. Human Genetics 1983;65:207-208.

119. Schwartz C, Fitch N, Phelan MC, Richer C-L, Stevenson R. Two sisters with a distal deletion at the Xq26/Xq27 interface: DNA studies indicate that the gene locus for factor IX is present. Human Genetics 1987;76:54-57.

120. Camerino G, Grzeschik KH, Jaye M, De La Salle H, Tolstoshev P, Lecocq JP, Heilig R, Mandel JL. Regional localization on the human X chromosome and polymorphism of the coagulation factor IX gene (haemophilia B locus). Proceedings of the National Academy of Sciences USA 1984;81:498-502.

121. Balland A, Faure T, Carvallo D. Characterisation of two differently processed forms of human recombinant factor IX synthesised in CHO cells transformed with a polycistronic vector. European Journal of Biochemistry 1988;172:565-571.

122. Furie B, Furie BC. The molecular basis of coagulation. Cell 1988;53:505-518.

123. Osterud KH, Laake K. Factor IX in warfarin treated patients. Thrombosis Research 1978;13:207-218.

124. Briët E, Tilburg NH, Veltkamp JJ. Oral contraception and the detection of carriers of haemophilia B. Thrombosis Research 1978;13:379-388.

125. Gralnick HR, Rick ME. Danazol increases factor VIII and factor IX in classic hemophilia and Christmas disease. The New England Journal of Medicine 1983;308:1393-1395.

126. Briët E, Wijnands MC, Veltkamp JJ. The prophylactic treatment of hemophilia B Leyden with anabolic steroids. Annals of Internal Medicine 1985;103:225-226.

127. Douglas AS, Mair K. The Christmas factor deficiency in coumarin therapy. Clinical Science 1958;17:445-455.

128. Denson KW. The levels of blood coagulation factors during anticoagulant therapy with phenindione. British Medical Journal 1961;1:1205-1210.

129. Edgell C-JS, Kirkman HN, Clemons E, Buchanan PD, Miller PH. Prenatal diagnosis by linkage: haemophilia A and polymorphic glucose-6-phosphate dehydrogenase. American Journal of Human Genetics 1978;30:80-84.

130. Renwick JH. Progress in mapping human autosomes. British Medical Bulletin 1969;25:65-73.

131. Kan YW, Dozy AM. Polymorphism of DNA sequence adjacent to human ß-globin structural gene: Relationship to sickle mutation. Proceedings of the National Academy of Sciences USA 1978;75:5631-5635.

132. Abbs S, Roberts RG, Mathew CG, Bentley DR, Bobrow M. Accurate assessment of intragenic recombination frequency within the Duchenne muscular dystrophy gene. Genomics 1990;7(4):602-606.

133. Cooper DN, Schmidke J. DNA restriction fragment length polymorphisms and heterozygosity in the human genome. Human Genetics 1984;66:1-16.

134. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 1975;98:503-517.

135. Winship PR, Anson DS, Rizza CR, Brownlee GG. Carrier detection in haemophilia B using two further intragenic restriction fragment length polymorphisms. Nucleic Acids Research 1984;12(23):8861-8872.

136. Freedenburg DL, Chen S-H, Kurachi K, Scott CS. Msp I polymorphic site within the factor IX gene. Human Genetics 1987;76:262-264.

137. Camerino G, Oberlé I, Drayna D, Mandel JL. A new Msp I restriction fragment length polymorphism in the haemophilia B locus. Human Genetics 1985;7:79-81.

138. Hay CW, Robertson KA, Yong S-L, Thompson AR, Growe GH, MacGillivray RTA. Use of a BamH I polymorphism in the factor IX gene for the determination of Haemophilia B carrier status. Blood 1986;67(5):1508-1511.

139. Driscoll MC, Dispenzieri A, Tobias E, Miller CH, Aledort LM. A second BamH I DNA polymorphism and haplotype association in the Factor IX gene. Blood 1988;72(1):61-65.

140. Winship PR, Brownlee GG. Diagnosis of haemophilia B carriers using intragenic oligonucleotide probes. Lancet 1986;ii:218-219.

141. Bowen DJ, Thomas P, Webb CE, Bignell P, Peake IR, Bloom AL. Facile and rapid analysis of the DNA polymorphisms within the human factor IX gene using the polymerase chain reaction. British Journal of haematology 1991;77:559-560.

142. Zhang M, Chen S-H, Scott CR, Thompson AR. The factor IX BamH I polymorphism: CpG transversion at the nucleotide sequence - 561. Human Genetics 1989;82:283-284.

143. Tsang TC, Bentley DR, Nilsson IM, Giannelli F. The Use of DNA Amplification for Genetic Counselling Related Diagnosis in Haemophilia B. Thrombosis and Haemostasis 1989;61(3):243-347.

144. Winship PR, Rees DJG, Alkan M. Detection of polymorphisms at Cytosine Phosphoguanadine dinucleotides and diagnosis of Haemophilia B carriers. Lancet 1989;i:631-634.

145. Graham JB, Kunkel GR, Egilmez NK, Wallmark A, Fowlkes DM, Lord ST. The varying frequencies of five DNA polymorphisms of X-linked coagulant Factor IX in eight groups. American Journal of Human Genetics 1991; 49:537-544.

146. Kojima T, Tanimoto M, kamiya T, Obata Y, Takahashi T, Ohno R, Kurachi K, Saito H. Possible absence of common polymorphisms in coagulation factor IX gene in Japanese subjects. Blood 1987;69:349-352.

147. Lubahn DB, Lord ST, Bosco J, Kirshtein J, Jeffries OJ, Parker N, Levstow C, Silverman LM, Graham JB. Population genetics of coagulation factor IX: Frequencies of two DNA polymorphisms in five ethnic groups. American Journal of Human Genetics 1987;40:527-536.

148. Cullen CR, Hubberman P, Kaslow DC, Migeon BR. Comparison of factor IX methylation on human active and inactive X-chromosomes: implications for X inactivation and transcription of tissue-specific genes. EMBO J 1986;2223-2229.

149. Smith KJ, Thompson AR, McMullen BA, Frazier D, Lin S-W, Stafford D, Kisiel W, Thibodeau SN, Chen S-H, Smith LF. Carrier testing in haemophilia B with an immunoassay that distinguishes a prevalent factor IX dimorphism. Blood 1987;70:1006-1013.

150. Arveiler B, Oberlé I, Vincent A, Hofker MH, Pearson PL, Mandel JL. Genetic mapping of the Xq27-q28 region: new RFLP markers useful for diagnostic applications in fragile-X and hemophilia-B families. American Journal of Human Genetics 1988;42(2):380-389.

151. Tanimoto M, Kojima T, Ogata K, Hamaguchi M, Takamatsu J, Kamiya T, Saito H. Extragenic factor IX gene RFLP is useful for detecting carriers of Japanese hemophilia B. Nippon Ketsueki Gakkai Zasshi 1989;52(4):774-777.

152. Van-der-Water NS, Ridgway D, Ockelford PA. Restriction fragment length polymorphisms associated with the factor VIII and factor IX in Polynesians. Journal of Medical Genetics 1991;28:171-176.

153. Mulligan L, Holden JJA, White BN. A DNA marker closely linked to the factor IX (Haemophilia B) gene. Human Genetics 1987;75:381-383.

154. Taylor SA, Lillicrap DP, Blanchette V, Giles AR, Holden JJA, White BN. A complete deletion of the factor IX gene and new Taq I variant in a hemophilia B kindred. Human Genetics 1988;79(3):273-276.

155. Sood R, Mulligan LM, Poon R, White BN, Holden JJ. Genetic mapping of two new DNA markers in Xq26-q28 relative to the fragile-X syndrome locus. American Journal of Human Genetics 1990;47(3):395-402.

156, Chung KS, Madar DA, Goldsmith JC, Kingdon HS, Roberts HR. Purification and characterization of an abnormal factor IX (Christmas factor) molecule: factor $IX_{Chapel\ Hill}$. Journal of Clinical Investigation 1978;62:1078-1085.

157. Noyes CM, Griffith MJ, Roberts HR, Lundblad RL. Identification of the molecular defect in factor $IX_{Chapel\ Hill}$: Substitution of histidine for arginine at position 145. Proceedings of the National Academy of Sciences USA 1983;80:4200-4202.

158. Giannelli F, Green PM, High KA, Lozier JN, Lillicrap DP, Ludwig M, Olek K, Reitsma PH, Goossens M, Yoshioka A, Sommer S, Brownlee GG. Haemophilia B: database of point mutations and short additions and deletions. Nucleic Acids Research 1990;18(14):4053-4059.

159. Green PM, Montandon AJ, Bentley DR, Ljung R, Nilsson IM, Giannelli F. The incidence and distribution of CpG----TpG transitions in the coagulation factor IX gene. A fresh look at CpG mutational hotspots. Nucleic Acids Research 1990;18(11):3227-31.

160. Bird AP. DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Research 1980:8:1499-1504.

161. Barker D. Schafer M, White R. Restriction Sites Containing CpG Show a Higher Frequency of Polymorphisms in Human DNA. Cell 1984;36:131-138.

162. Giannelli F, Choo KH, Rees DJG, Boyd Y, Rizza CR, Brownlee GG. Gene deletions in patients with haemophilia B and anti-factor IX antibodies. Nature 1983;303:181-182.

163. Matthews RJ, Anson DS, Peake IR, Bloom AL. Heterogeneity of the factor IX locus in nine hemophilia B inhibitor patients. Journal of Clinical Investigation 1987;79:746-753.

164. Anson DS, Blake DJ, Winship PR, Birnbaum D, Brownlee GG. Nullisomic deletion of mcf2 transforming gene in two haemophilia B patients. EMBO 1988;7:2795-2799.

165. Green PM, Bentley DR, Mibashan RS, Giannelli F. Partial deletion by illegitimate recombination of the factor IX gene in a haemophilia B family with two inhibitor patients. Molecular Biology and Medicine 1987;5:95-106.

166. Bray GL, Thompson AR. Partial factor IX protein in a pedigree with hemophilia B due to a partial gene deletion. Journal of Clinical Investigation 1986;77:1194-1200.

167. Vidaud M, Chabret C, Gazengel C, Grunebaum L, Cazenave JP, Goossens M. A *de novo* intragenic deletion of the potential EGF domain of the factor IX gene in a family with severe haemophilia B. Blood 1986;68(4):961-963.

168. Giannelli F. Advances in the molecular biology of coagulation factor IX and their practical implications. In: Giovannucci Uzielli ML, Tavellini F, Bussani C, Guarducci S (eds.) Proceedings of the International Symposium: "From Man to Gene, From Gene to Man." Florence 1986, pp122-129.

169. Casarino L, Sangigorgi S, Pecorara M. Carrier detection of haemophilia B with factor IX DNA specific probe. Preliminary report. In: Ciavarella NL, Ruggeri ZM, Zimmerman TS (eds). Factor VIII/von Willebrand Factor: Biological and Clinical Advances, Milan. pp210-205.

170. Tanimoto M, Kojima T, Kamiya T, Takamatsu J, Ogata K, Obata Y, Inagaki M, Iisuka A, Nagao T, Kurachi K, Saito H. DNA analysis of seven patients with haemophilia B who have anti-factor IX antibodies: relationship to clinical manifestations and evidence that the abnormal gene was inherited. Journal of Laboratory and Clinical Medicine 1988;112:307-313.

171. Taylor SAM, Lillicrap DP, Blanchette V, Giles AR, Holden JJA, White BN. A complete deletion of the factor IX gene and new Taq I variant in a haemophilia B kindred. Human Genetics 1988;79:273-276.

172. Ludwig M, Schaab R, Brackmann HH, Eali H, Olek K. Molecular defects of the factor IX gene causing severe haemophilia B. Thrombosis and Haemostasis 1987;58:352.

173. McGraw RA, Davis LM, Lundblad RL, Roberts HR, Graham JB, Stafford DW. Structure and function of factor IX: defects in haemophilia B. Clinical Haematology 1985;14:359-383.

174. Mikamai S, Nishino M, Nishimura T, Fukui H. RFLP's of factor IX gene in Japanese haemophilia B families and gene deletion in two high responder-inhibitor patients. Japanese Journal of Human Genetics 1987;32:21-31.

175. Chen SH, Scott CR, Edson JR, Kurachi K. An insertion within the factor IX gene: Haemophilia $B_{El\ Salvador}$. American Journal of Human Genetics 1988;42:581-584.

176. Briët E, Bertina RM, van Tiblburg NH, Veltkamp JJ. Hemophilia B Leyden: A sex-linked hereditary disorder that improves after puberty. New England Journal of Medicine 1982;306:788-790.

177. Reitsma PH, Bertina RM, Ploos van Amstel JK, Riemens A, Briët E. The Putative Factor IX Gene Promoter in Hemophilia B Leyden. Blood 1988;72(3):1074-1076.

178. Reitsma PH, Mandalaki T, Kasper C, Bertina RM, Briët E. Two Novel Point Mutations Correlate with an Altered Developmental Expression of Blood Coagulation Factor IX (Hemophilia B Leyden Phenotype). Blood 1989;73(3):743-746.

179. Gitschier J, Wood WI, Goralka TM, Wion KL, Chen EY, Eaton DH, Vehar GA, Capon DJ, Lawn RM. Characterization of the human factor VIII gene. Nature 1984;312:326-330.

180. Toole JJ, Knopf JL, Wozney JM, Sultzman LA, Buecker JL, Pittman DD, Kaufman RJ, Brown E, Shoemaker C, Orr EC, Amphlett GW, Foster WB, Coe ML, Knutson GJ, Fass DN, Hewick RM. Molecular cloning of a cDNA encoding human antihaemophilic factor. Nature 1984;312:342-347.

181. Tantravahi U, Murty VV, Jhanwar SC, Toole JJ, Woozney JM, Chaganti RS, Latts SA. Physical mapping of the factor VIII gene proximal to two polymorphic DNA probes in human chromosome band Xq28: implications for factor VIII gene segregation analysis. Cytogenetics and Cell Genetics 1986;42:75-79.

182. Vehar GA, Keyt B, Eaton D, Rodriguez H, O'Brien DP, Rotblat F, Oppermann H, Keck R, Wood WI, Harkins RN, Tuddenham EGD, Lawn RM, Capon DJ. Structure of human factor VIII. Nature 1984;312:337-342.

183. Wood, WI, Capon, DJ, Simonsen, CC, Eaton, DL, Gitschier, J, Keyt, B, Seeburg, PH, Smith, DH, Hollingshead, P, Wion, KL, Delwart, E, Tuddenham, EGD, Vehar, GA, Lawn, RM. Expression of active human factor VIII from recombinant DNA clones. Nature 1984;312:330-337.

184. Webster WP, Zukoski CF, Hutchin P, Reddick RL, Mandel SR, Penick GP. Plasma factor VIII synthesis and control as revealed by canine organ transplantation. American Journal of Physiology 1971;220:1147-1153.

185. Wion KL, Kelly D, Summerfield JA, Tuddenham EGD, Lawn RM. Distribution of factor VIII mRNA and antigens in human liver and other tissues. Nature 1989;317:726-729.

186. Kadhom N, Wolfrom C, Gautier M, Allain JP, Frommel D. Factor VIII procoagulant antigen in human tissues. Thrombosis and Haemostasis 1988;59:289-294.

187. Kobrinsky NL, Watson CM, Cheang MS, Bishop AJ. Improved haemophilia A carrier detection by DDAVP stimulation of factor VIII. Journal of Pediatrics 1984;104(5):718-724.

188. Mannucci PM, Ruggeri ZM, Pareti FL, Capitanio A. 1-Deamino-8-D-arginine vasopressin: A new pharmacological approach to the management of hemophilia and von Willebrand's disease. Lancet 1977;i:869-872.

189. de la Fuente B, Kasper CK, Rickles FR, Hoyer LW. Response of patients with mild and moderate hemophilia A and von Willebrand's disease to treatment with vasopressin. Annals of Internal Medicine 1985;103:6-14.

190. Harper K, Pembrey ME, Davies KE, Winter RM, Hartley D, Tuddenham EGD. A clinically useful DNA probe closely linked to haemophilia A. Lancet 1984;ii:6-7.

191. Oberlé I, Camerino G, Helig R, Grunebaum L, Cazenave JP, Crapanzano C, Mannucci PM, Mandel JL. Genetic screening for haemophilia A (classic haemophilia) with a polymorphic DNA probe. New England Journal of Medicine 1985;312:682-686.

192. Peake IR. Registry of DNA Polymorphisms Within or Close to the Human Factor VIII and Factor IX Genes. Thrombosis and Haemostasis 1992; 67(2):277-280.

193. Gitschier J, Drayna D, Tuddenham EGD, White RL, Lawn RM. Genetic mapping and diagnosis of haemophilia A achieved through a Bcl I polymorphism in the factor VIII gene. Nature 1985;314:738-740.

194. Antonarakis SE, Waber PG, Smita MS, Kittur SD, Patel AS, Kazazian HH, Mellis MA, Counts RB, Stamatoyannopoulos G, Bowie EJW, Fass DN, Pittman DD, Wozney JM, Toole JJ. Haemophilia A - detection of molecular defects and of carriers by DNA analysis. New England Journal of Medicine 1985;313(14):842-848.

195. Wion KL, Tuddenham EGD, Lawn RM. A new polymorphism in the factor VIII gene for prenatal diagnosis of haemophilia A. Nucleic Acids Research 1986;14(11):4535-4542.

196. Ahrens P, Kruse TA, Schwartz M, Rasmussen PB, Din N. A new Hind III restriction fragment length polymorphism in the hemophilia A locus. Human Genetics 1987;26:127-128.

197. Youssoufian H, Phillips DG, Kazazian HH, Antonarakis SE. Msp I polymorphism in the 3' flanking region of the human FVIII gene. Nucleic Acids Research 1987;15(15):6312.

198. Inaba H, Fujimaki M, J.J. K, Antonarakis SE. Msp I polymorphism site in intron 22 of the factor VIII gene in the Japanese Population. Human Genetics 1990;84:214-215.

199. Jedlica P, Greer S, Millar DS, Grundy CB, Jenkins E, Mitchell M, Mibashan RS, Kakkar VV, Cooper DN. Improved carrier detection of haemophilia A using novel RFLP's at the DXS115 (767) locus,. Human Genetics 1990;85:315-318.

200. Paterson MN, Gitschier J, Bloomfield J, Bell M, Dorkins H, Froster-Iskenius U, Sommer S, Sobell J, Schaid D, Thibodeau S, Davies KE. An intronic region within the human FVIII gene is duplicated within Xq29 and is homologous to the polymorphic locus DXS115 (767). Human Genetics 1989;44:679-685.

201. Kenwick S, Bridge P, Lillicrap D, Lehesjoki AE, Bainton J, Gitschier J. A Taq I polymorphism adjacent to the factor VIII gene. Nucleic Acids Research 1991;19:2513.

202. Kogan SC, Doherty M, Gitschier J. An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. New England Journal of Medicine 1987;317(16):985-990.

203. Graham JB, Kunkel GR, Fowlkes DN, Lord ST. The utility of a Hind III polymorphism of factor VIII examined by rapid DNA analysis. British Journal of Haematology 1990;76:75-79.

204. Richards B, Heilig R, Oberlé I, Storjohann L, Hort GT. Rapid PCR analysis of the ST14 (DXS52) VNTR. Nucleic Acids Research 1991;91:1944.

205. Kogan SC, Gitschier J. Detection of hemophilia A mutations near the acidic region of factor VIII by DNA amplification and denaturing gradient gel electrophoresis. Blood 1988;72:300.

206. Lalloz MRA, McVey JH, Pattinson JK, Tuddenham EGD. Haemophilia A diagnosis by analysis of a hypervariable dinucleotide repeat within the factor VIII gene. Lancet 1991;338:207-211.

207. Chan V, Chan TK, Lui VWS, Wong ACK. Restriction fragment length polymorphisms associated with factor VIII:C gene in Chinese. Human Genetics 1988;79:128-131.

208. Suehiro K, Tanimoto M, Hamaguchi M, Kojima T, Takamatsu J, Ogata K, Kamiya T, Saito H. Carrier detection in Japanese hemophilia A by use of three intragenic and two extragenic factor VIII DNA probes: a study of 24 kindred. The Journal of Laboratory and Clinical Medicine 1988;112(3):314-318.

209. Van der Water NS, Ridgway D, Ockelford PA. Restriction fragment length polymorphisms associated with the factor VIII and factor IX genes in Polynesians. Journal of Medical Genetics 1991;28(3):171-176.

210. Tuddenham EGD, Cooper DN, Gitschier J, Higuchi M, Hoyer LW, Yoshioka A, Peake IR, Schwaab R, Olek K, Kazazian HH, Lavergne J-L, Giannelli F, Antonarakis SE. Haemophilia A: database of nucleotide substitutions, deletions, insertions and rearrangements of the factor VIII gene. Nucleic Acids Research 1991;19(18):4821-4833.

211. Higuchi M, Kazazian HJ, Kasch L, Warren TC, McGinniss MJ, Phillips J, Kasper C, Janco R, Antonarakis SE. Molecular characterization of severe hemophilia A suggests that about half the mutations are not within the coding regions and splice junctions of the factor VIII gene. Proceedings of the National Academy of Sciences U S A 1991;88(16):7405-7409.

212. Gitschier J, Wood WI, Tuddenham EGD, Shuman MA, Goralka TM, Chen EY, Lawn RM. Detection and sequence of mutations in the factor VIII gene of haemophiliacs. Nature 1985;315:427-430.

213. Antonarakis SE. Hemophilia A persistence and gene mutational vulnerability. Hospital Practice 1987;22:93-98.

214. Youssoufian H, Antonarakis SE, Aronis S, Tsiftis G, Phillips DG, Kazazian HH. Characterization of five partial deletions of the factor VIII gene. Proceedings of the National Academy of Sciences USA 1987;84:3772-3776.

215. Higuchi M, Kochlan L, Schwaab R, Egli H, Brackman HE, Horst J, Olek K. Molecular Defects in Hemophilia A: Identification and Characterization of Mutations in the Factor VIII Gene and Family Analysis. Blood 1989;74(3):1045-1051.

216. Higuchi M, Kochhan L, Olek K. A somatic mosaic for Haemophilia A detected at the DNA level. Molecular Biology in Medicine 1988;5:23-27.

217. Higuchi M, Kochlan L, Schwaab R, Brackman HH, Egli H, Olek K. Detection of mutations of hemophilia A. Thrombosis and Haemostasis 1987;58:336.

218. Din N, Schwartz M, Kruse T, Vestegaard SR, Ahrens OP, Scheibel E, Nordfang O, Ezban M. Factor VIII gene-specific probes used to study heritage and molecular defects in hemophilia A. La Ricerca in Clinica e in Laboratorio 1986;16:227.

219. Casarino L, Pecorara M, Mori PG, Morfini M, Mancuso G, Scrivano L, Molinari AC, Lanza T, Giavarella G, Loi A, Perseu L, Cao A, Pirastu M. Molecular basis for hemophilia A in Italians. La Ricerca in Clinica e in Laboratorio 1986;16:227.

220. Bardoni B, Sampietro M, Romano M, Crapanzano M, Mannucci PM, Camerino G. Characterization of a partial deletion of the factor VIII gene in a haemophiliac with inhibitor. Human Genetics 1988;79:86-88.

221. Lillicrap DP, Taylor SAM, Grover H, Teitel J, Giles AR, Holden JJA. Genetic analysis in haemophilia A: identification of a large FVIII gene deletion in a patient with high titre antibodies to human and porcine FVIII. Blood 1986;68:337.

222. Bernardi F, Volinia S, Patracchini P, Gemmati D, Boninsegna S, Schwienbacher C, Marchetti G. A recurrent missense mutation (Arg-Gln) and a partial deletion in factor VIII gene causing severe haemophilia A. British Journal of Haematology 1889;71:271-276.

223. Gitschier J. Maternal duplication associated with gene deletion in sporadic Haemophilia. American Journal of Genetics 1988;43:274-279.

224. Kazazian HH, Wong C, Youssoufian H, Scott AF, Phillips DG, Antonarakis SE. Haemophilia A resulting from *de novo* insertion of L1 sequences represents a novel mechanism for mutation in man. Nature 1988;332:164-167.

225. Youssoufian H, Kazazian HH, Patel A. Mild hemophilia A associated with a cryptic donor spice site mutation in intron 4 of the factor VIII gene. Genomics 1988;2:32-36.

226. Youssoufian H, wong C, Aronis S, Platokoukis H, Kazazian HH, Antonarakis SE. Moderately severe haemophilia A resulting from Glu-Gly substitution in exon 7 of the factor VIII gene. American Journal of Human Genetics 1988;42:867-871.

227. Gitschier J, Kogan S, Levinson B, Tuddenham EGD. Mutations of Factor VIII cleavage sites in Haemophilia A. Blood 1988;72(3):1022-1028.

228. Pattinson JK, McVey JH, Boon M, Avani A, Tuddenham EGD. CRM^+ Haemophilia A due to a missense mutation (372-Cys) at the internal heavy chain thrombin cleavage site. British Journal of Haematology 1990;75:73-77.

229. Fulcher CA, Mahoney S de G, Roberts JR, Kasper CK, Zimmerman TS. Localization of human factor VIII inhibitor epitopes to two polypeptide fragments. Proceedings of the National Academy of Sciences USA 1985;82:7728-7732.

230. Levinson B, Janco R, Phillips J, Gitschier J. A novel missense mutation in the factor VIII gene identified by analysis of amplified hemophilia DNA sequences. Nucleic Acids Research 1988;15:9797-9805.

231. Youssoufian H, Antonarakis SE, Bell W, Griffin AM, Kazazian HH. Nonsense and missense mutations in hemophilia A: estimate of the relative mutation rate at CG dinucleotides. American Journal of Human Genetics 1988;42:718-725.

232. Bernardi F, Volinia S, Patracchini P, Gemmati D, Boninsegna S, Schwienbacvher C, Marchetti G. A recurrent missense mutation (Arg-Gln) and a partial deletion in factor VIII gene causing severe haemophilia A. British Journal of Haematology 1989;71:271-276.

233. Bernardi F, Legnani C, Volinia S, Patracchini P, Rodorigo G, DeRosa V, Marchetti G. A Hind III RFLP and a gene lesion in the coagulation factor VIII gene. Human Genetics 1988;78:359-362.

234. Gitschier J, Wood WI, Shuman MA, Lawn RM. Identification of a missense mutation in the factor VIII gene of a mild haemophiliac. Science 1986;232:326-330.

235. Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Research 1988;16:11141-11156.

236. Myers RM, Larin Z, Maniatis T. Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. Science 1985;230:1242-1246.

237. Myers RM, Lumelsky N, Lerman LS, Maniatis T. Detection of single base substitutions in total genomic DNA. Nature 1985;313:495-498.

238. Cotton RG, Rodrigues NR, Campbell RD. Reactivity of cytosine and thymine in single-base pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. Proceedings of the National Academy of Sciences USA 1988;85:4397-4401.

239. Newton CR, Kalsheker N, Graham A, Powell S, Gammack A, Markham AF. Diagnosis of α_1-antitrypsin deficiency by enzymatic amplification of human genomic DNA and direct sequencing of polymerase chain reaction products. Nucleic Acids Research 1988;16:8233-8234.

240. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. Science 1985;230:1350-1354.

241. Newton CR, Graham A, Hepinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Research 1989;17(7):2503-2516.

242. Montandon AJ, Green PM, Giannelli F, Bentley DR. Direct detection of point mutations by mismatch analysis: application to haemophilia B. Nucleic Acids Research 1989;17(9):3347-3358.

243. Lyon M. Sex chromatin and gene action in the mammalian X-chromosome. American Journal of Human Genetics 1962;14:135-148.

244. Nisen PD, Waber PG. Non-random X-chromosome DNA methylation patterns in Hemophilic females. Blood (Suppl.1) 1988;72:1127.

245. Akhmeteli MA, Aledort IM, Akexabuabs S, Bulanov AG, Elston RC, Ginter EK, Goussey A, Graham JB, Hermans J, Larrieu MJ, Lothe F, Mclaren AD, Mannucci PM, Prentice CRM, Veltkamp JJ. Methods for the detection of haemophilia carriers: A memorandum. Bulletin of the World Health Organization 1977;55:675-702.

246. Graham JB, Rizza CR, Chediak J, Mannucci PM, Briët E, Ljung R, Kasper CK, Essien EM, Green PP. Carrier detection in Haemophilia A: A Cooperative International Study. I. The carrier phenotype. Blood 1986;67(6):1554-1559.

247. Stableforth P, Montgomery DC, Wilson E, Churchill WGL, Dormandy K, Hardisty RM. Effect of oral contraceptives on factor VIII clotting activity and factor VIII related antigen in normal women. Journal of Clinical Pathology 1975;28:498-501.

248. Schloessmann H. Die Hämophilie, Neue deutsche Chirhgie, Stuttgart 1930, Ferdinand Enke.

249. Andreassen M. Opera ex Domo Biologiae Hereditariae Humanae, Universitatis Hafniensis (Hamofili I Danmark). Ejnar Munksgaard, Kobenhavn, 1943.

250. Sköld E. On Hemophilia in Sweden and its treatment by blood transfusion. Acta Medica Scandinavica 1944;150 (Suppl.1):1-247.

251. Merskey C, Macfarlane RG. The female carrier of haemophilia: A clinical and laboratory study. Lancet 1951;i:487-490.

252. Graham JB, Mclendon WW, Brinkhous KM. Mild hemophilia: an allelic form of the disease. American Journal of Medical Science 1953;225:46-53.

253. Gardiakas C, Katsiroumbas P, Kottas C. The Antihemophilic Globulin Concentration in the plasma of Female Carriers of Haemophilia. British Journal of Haematology 1957;3:377-378.

254. Margolius A, Ratnoff OD. A laboratory study of the carrier state in classic hemophilia. Journal of Clinical Investigation 1956;35:1316-1323.

255. Zimmerman TS, Ratnoff OD, Littell AS. Detection of carriers of classic haemophilia using an immunologic assay for antihaemophilic factor (factor VIII). Journal of Clinical Investigation 1971;50:255-258.

256. Ratnoff OD, Jones PK. The laboratory diagnosis of the carrier state for classic haemophilia. Annals of Internal Medicine 1977;86(5):521-528.

257. Seligshon U, Zivelin A, Perez C, Modan M. Detection of haemophilia A carriers by replicate factor VIII activity and factor VIII antigenicity determinations. British Journal of Haematology 1979;42:433-439.

258. Graham JB, Barrow ES, Lyer P, Dawson DV, Elston RC. Identifying carriers of Mild haemophilia. British Journal of Haematology 1980;44:671-679.

259. Marshall LR, Stenhouse NS. Triple parameter discriminant analysis and carriers of haemophilia. Thrombosis and Haemostasis 1979;42:810-811.

260. Shen MC. A comparative study of carrier detection in haemophilia A by linear discriminant function. British Journal of Haematology 1982;52:283-293.

261. Duncan BM, Tunbridge LJ, Duncan EM, Lloyd JV. Detection of haemophilia carriers: multivariate analysis compared with discriminant analysis using up to five factor VIII variables. British Journal of Haematology 1984;57:113-121.

262. Hoyer LW, Carta CA, Mahoney MJ. Detection of haemophilia carriers during pregnancy. Blood 1982;60(6):1407-1410.

263. Douglas AS, Cook IA. Deficiency of Antihemophilic Globulin in Heterozygous Female Haemophilic Females. Lancet 1957;ii:616-619.

264. Taylor K, Biggs R. A mildly affected female haemophiliac. British Medical Journal 1957;1:1494-1496.

265. Didisheim P, Ferguson JH, Lewis JH. Hemostatic data in relatives of hemophiliacs A and B; evidence for modifying the classical sex-linked recessive hypothesis. Journal of the American Medical Association 1958;101:347-354.

266. Pitney WR, Arnold BJ. Plasma antihaemophilic factor (AHF) concentrations in families of Patients with Haemorrhagic states. British Journal of Haematology 1959;5:184-193.

267. Ikkala E. Haemophilia. A study of its laboratory, clinical, genetic and social aspects based on known haemophiliacs in Finland. Scandinavian Journal of Clinical Investigation 1960;12:Supplement 46.

268. Bentley HP, Krivit W. An assay of antihemophilic globulin activity in the carrier female. Journal of Laboratory and Clinical Medicine 1960;56:613-621.

269. Rapaport SI, Patch MJ, Moore FJ. Anti-hemophilic globulin levels in carriers of hemophilia A. Journal of Clinical Investigation 1960;39:1619-1625.

270. Githens JH, Wilcox PJ. The carrier state in hemophilia A. Journal of Pediatrics 1962;60:77-83.

271. Nilsson IM, Blombäck M, Ramgreen O, Francken IV. Hemophilia in Sweden. II - Carriers of Hemophilia A and B. Acta Medica Scandinavica 1962;171:223-235.

272. Goudemand MM, Foucant A, Hutin A, Parquet-Gernez G, Mackey-Bermelle G. Les conductices de l'hémophilie A. Lille Méd 1962;7:469.

273. Bradlow BA. Antihaemophilic globulin levels in female carriers of haemophilia. South African Journal of Medical Science 1962;27(3):51-55.

274. Deutsch E, Kock M. Beitrag zur Objektivierung der Diagnose der hämophilen Konduktorin. Wein Klin Wschr 1962;74:793.

275. Mulder E, Mochtar IA, van Creveld S, Lopes Cardozo EB. Factor VIII activity in carriers of haemophilia A. British Journal of Haematology 1965;ii:206-209.

276. Miller SP, Siggerud J. Abnormal blood coagulation in carriers of hemophilia. Journal of Laboratory and Clinical Medicine 1963;63:621-637.

277. Kerr DB, Preston AE, Barr A, Biggs R. Further studies on the inheritance of Factor VIII. British Journal of Haematology 1966;12:212-233.

278. Gugler E, Rosin S, Bütler R. Gerinnungsphysiologische Untersuchungen bei heterozygoten Anlaget.

279. Bergna LJ, Pavlovsky A. Concentration of Factor VIII in the blood of mothers of patients with hemophilia. Proceedings of the 9th. International Congress in Blood Transfusion. Mexico 1964, p.161.

280. Bennett E, Huehns ER. Immunological differentiation of three types of Haemophilia and identification of some female carriers. Lancet 1970;ii:956-958.

281. Ekert H, Helliger H, Muntz RH. Detection of Carriers of Haemophilia. Thrombosis et Diathesis Haemorrhagica 1973;30:255-262.

282. Bennett B, Ratnoff OD. Detection of the carrier state for classic hemophilia. New England Journal of Medicine 1973;288(7):342-345.

283. Denson KWE. The Detection of Factor-VIII-Like Antigen in Haemophilic Carriers and in Patients with Raised Levels of Biologically Active Factor VIII. British Journal of Haematology 1973;24:451-461.

284. Bouma BN, Van der Klaauw MM, Veltkamp JJ, Starkenburg AE, Van Tilburg NH, Hermans J. Evaluation of the detection rate of hemophilia carriers. Thrombosis Research 1975;7:339-350.

285. Meyer D, Plas A, Allain JP, Sitar GM, Larrieu MJ. Problems in the detection of carriers of haemophilia A. Journal of Clinical Pathology 1975;28:690-695.

286. Eyster ME, Jones MB, Moore T, Delli-Bovi L. Carrier Detection in Classic Hemophilia by Combined Measurement of Immunologic (VIII AGN) and Procoagulant (VIII AHF) Activities. American Journal of Clinical Pathology 1976;65:9754-981.

287. Rizza CR, Rhymes IL, Austen DEG, Kernoff OBA, Arnoi SA. Detection of Carriers of Haemophilia: a "Blind" Study. British Journal of Haematology 1975;30:447-456.

288. Prentice CRM, Forbes CD, Morrice S. Calculation of predictive odds for possible carriers of haemophilia. In: Ulutin ON, Peake IR (eds.) Haemophilia. American Elsevier Publishing Co., New York 1975, pp 40-46.

289. Hoyer LW. Immunologic studies on antihemophilic factor (AHF, factor VIII). IV. Radioimmunoassay of AHF antigen. Journal of Laboratory and Clinical Medicine 1972;80:822-833.

290. Graham JB, Miller CH, Reisner HM, Elston RC, Olive JA. The Phenotypic Range of Hemophilia A Carriers. American Journal of Human Genetics 1976;28:482-488.

291. Hathaway HS, Lubs ML, Kimberling WJ, Hathaway WE. Carrier Detection in Classical Hemophilia. Pediatrics 1976;57(2):251-254.

292. Weinstein MJ, Deykin D, Davie EW. Quantitative determination of factor-VIII protein by two-stage gel electrophoresis. British Journal of Haematology 1976;33:345-355.

293. Gomperts ED, Fatti LP, van der Walt JD. Factor VIII and factor VIII-related antigen in normal South African blacks and a black carrier group. Thrombosis Research 1976;9:293-299.

294. Klein HG, Aledort LM, Bouma BN, Hoyer LW, Zimmerman TS, DeMets DL. A co-operative study for the detection of the carrier state of classic haemophilia. New England Journal of Medicine 1977;296(17):959-962.

295. Ratnoff OD, Jones PK. The laboratory diagnosis of the carrier state for classic haemophilia. Annals of Internal Medicine 1977;86(5):521-528.

296. Peake IR, Newcombe RG, Davies BL, Furlong RA, Ludlam CA, Bloom AL. Carrier detection in haemophilia A by immunological measurement of factor VIII related antigen (VIIIR:Ag) and factor VIII clotting antigen (VIIIC:Ag). British Journal of Haematology 1981;48:651-660.

297. Mibashan RS, Rodeck CH, Nicolaides KH, Warren R, Pembrey ME, Giannelli F. Phenotypic and DNA diagnosis of fetal bleeding disorders. Thrombosis and Haemostasis 1981;46:187.

298. Wahlberg T, Blombäck M, Brodin U. Carriers and non-carriers of haemophilia A: I. Multivariate analysis of pedigree data, screening blood coagulation tests and factor VIII variables. Thrombosis Research 1982;25:401-414.

299. Green PP, Mannucci PM, Briët E, Ljung R, Kasper CK, Essien EM, Chediak J, Rizza CR, Graham JB. Carrier detection in haemophilia A: a cooperative international study. II. The efficacy of a universal discriminant. Blood 1986;67(6):1560-1567.

300. Graham JB, Rizza CR, Chediak J, Mannucci PM, Briët E, Ljung R, Kasper CK, Essien EM, Green PP. Carrier detection in Haemophilia A: A cooperative international study. I. The carrier phenotype. Blood 1986;67(6):1554-1559.

301. Hellstern P, Miyasita C, Köhler M. G, Kiehl, R, Biro, G, Schwerdt, H, Wenzel, E, 1987. Measurement of factor VIII procoagulant antigen in normal subjects and in haemophilia A patients by an immunoradiometric assay and by an enzyme-linked immunosorbent assay. Haemostasis 1987;17:173-181.

302. Percy ME, Rusk ACM, Garvey MB, Freedman J, Teitel JM, Blake P, Carter C, Andrew M, Johnson M, Inwood M, Andrews DF, Brasher PMA. Carrier detection in Hemophilia A: ABO blood group, multiple measurements and application of logistic discrimination. American Journal of Medical Genetics 1988;31:871-879.

303. Ridgway H, Gennings A, Speer RJ. Detection of the carrier state of hemophilia A in females. Wadley Medical Bulletin 1973:3:68-70.

304. Holmberg L, Nilsson IM, Stölten C. Fifteen-year follow-up of potential carriers of haemophilia A and their progeny. In: Haemophilia Ultin ON, Peake IR (eds.). American Elsevier Publishing Co., New York. 1975.

305. Panicucci F, Baicchi U, Sagripanti A, Pinori E, Bruno V. Detection of carriers of haemophilia. In: Ultin ON, Peake IR (eds.) Haemophilia. Excerpta Medica, Amsterdam, 1975 pp52-59.

306. Lusher JM, Young E, Kim C. Factor VIII antigen quantitation in the detection of 'carriers' of hemophilia and in the diagnosis of von Willebrand Disease. XIth Congress World Federation of Hemophilia 1976. p42.

307. Lian E.C-Y, Diez-Ewald M, Lian MT, Nunez RL, Harkness DR. Detection of hemophilic carriers by determination of the ratios between ristocetin cofactor activity and factor VIII (AHF) activity. 16th. International Congress of Hematology, Kyoto. 1976 Abstracts. p327.

308. Matsuzo M, Masakazu I, Takahashi K, Sakuragawa N. An immunological method for detection of the carrier of hemophilia B. Thrombosis and Haemostasis 1976;36:441-450.

309. Thompson AR. Factor IX Antigen by radioimmunoassay in heterozygotes for hemophilia B. Thrombosis Research 1977;11:193-203.

310. Kasper CK, Osterud B, Minami JY, Shonick W, Rapaport SI. Hemophilia B: characterisation of genetic variants and detection of carriers. Blood 1977;50(3):351-356.

311. Frota-Pessoa O, Gomes EL, Calicchio TR. Christmas factor: Dosage compensation and the production of blood coagulation factor IX. Science 1963;139:348-349.

312. Didisheim P, Vandervoot RLE. Detection of carrier for factor IX (PTC) deficiency. Blood 1962;20(2):150-155.

313. Elödi S. Factor IX activity and factor IX antigen in haemophilia B carriers. Thrombosis Research 1975;6:39-51.

314. Graham JB, Flyer P, Elston RC, Kasper CK. Statistical study of genotype assignment (carrier detection) in hemophilia B. Thrombosis Research 1979;15:69-78.

315. Ørstavik KH, Veltkamp JJ, Bertina RM, Herman J. Detection of carriers of haemophilia B. British Journal of Haematology 1979;42:293-301.

316. Firkin BG. The demonstration of the carrier state in Christmas Disease. The Medical Journal of Australia 1958;1:557-558.

317. Bond T, Cleander DR, Guest MM. The detection of Plasma Thromboplastin Component (PTC) deficiency carrier. Federation Proceedings 1959;18:14.

318. Bolton FG, Clarke JE. A method of assaying Christmas Factor; its application to the study of Christmas Disease (Factor-IX deficiency). British Journal of Haematology 1959;5:396-412.

319. Barrow EM, Bullock WR, Graham JB. A study of the carrier state for plasma thromboplastin component (PTC, Christmas factor) deficiency, utilizing a new assay procedure. Journal of Laboratory and Clinical Medicine 1960;55:936-945.

320. Simpson NE, Biggs R. The inheritance of Christmas factor. British Journal of Haematology 1962;8:191-203.

321. Gugler E, Rosin S, Bütler R. Gerinnungsphysiologische untersuchungen bei heterozygoten Anlageträgerinnen der Hämophilie. Schweiz med. Wschr 1965;10:320.

322. Bayes T. An essay towards solving a problem in the doctrine of chance. Biometrika 1958;45:296-315.

323. Young ID. Introduction to Risk Calculation in Genetic Counselling. Oxford University Press, 1991: p1-6.

324. Winter RM, Tuddenham EGD, Goldman E, Matthews KB. A maximum likelihood estimate of the sex ratio of mutation rates in haemophilia. Human Genetics 1983;64:156-159,.

325. Short PE, Williams CE, Picken AM, Hill FGH. Factor VIII related antigen: an improved enzyme immunoassay. Medical Laboratory Sciences 1982;39:351-355.

326. Goodall AH, Jarvis J, Chand S, Rawlings E, O'Brien DP, McGraw A, Hutton R, Tuddenham EGD. An immunoradiometric assay for human Factor VIII/von Willebrand Factor (VIII:vWF) using monoclonal antibodies that define a functional epitope. British Journal of Haematology 1985;59:565-577.

327. Dacie JV, Lewis SM. Practical Haematology. Churchill Livingstone, Edinburgh. 1984. pp. 232.

328. Bell GI, Karman JH, Rutter WJ. Polymorphic DNA region adjacent to the 5' end of the human insulin gene. Proceedings of the National Academy of Sciences USA 1981;78(9):5759-5763.

329. Maniatis T, Fritsch EF, Sambrook J, Eds. Molecular cloning: A laboratory manual. 1982, Cold Spring Harbour, Cold Spring Harbour Laboratory, NY.

330. Mandel M, Higa A. Calcium dependent bacteriophage infection. Journal of Molecular Biology 1970;53:159-162,

331. Cohen SN, Chang ACY and Hsu L. Non-chromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor. Proceedings of the National Academy of Sciences USA 1973;69(8):2110-2114.

332. Feinberg AP, Vogelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry 1983;132:6-13.

333. Feinberg AP, Vogelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry 1983;137:266-267.

334. Moodie P, Liddell MB, Peake IR, Bloom AL. Carrier detection in 50 haemophilia A kindred by means of three intragenic and two extragenic restriction fragment length polymorphisms. British Journal of Haematology 1988;70:77-84.

335. Bröcker-Vriends AHJT, Briët E, Quadt R, Dreesen JCFM, Bakker E, Claassen-Tegelaar R, Kanhai HHH, van de Kamp JJP, Pearson PL. Genotype Assignment of Haemophilia A by Use of Intragenic and Extragenic Restriction Fragment Length Polymorphisms. Thrombosis and Haemostasis 1987; (57(2):131-136.

336. Janco RL, Phillips JA, Orlando PJ, Woodard MJ, Wion KL, Lawn RM. Detection of hemophilia A carriers using intragenic factor VIII:C DNA polymorphisms. Blood 1987;69:1539-1541.

337. Migeon BR, Moser HW, Axelman J, Sillence D. Adrenoleukodystrophy: evidence for X-linkage, inactivation and selection favouring the mutant allele in heterozygous cells. Proceedings of the National Academy of Sciences USA 1981;78:5066-5070.

338. Auborg P, Feil R, Guidoux S, Kaplan J-C, Moser S, Mandel JL. The red-green pigment gene region in adrenoleukodystrophy. American Journal of Human Genetics 1990;46:459-469.

339. Auborg PR, Sack GH, Meyers DA, Lease JJ, Moser HW. Linkage of Adrenoleukodystrophy to a Polymorphic DNA Probe. Annals of Neurology 1987;21:349-352.

340. Auborg PR, Sack GH, Moser HW. Frequent alteration of visual pigment gene in adrenoleukodystrophy. American Journal of Human Genetics 1988;42:408-413.

341, Youssoufian H, Kazazian HH, Phillips DG, Aronis S, Tsiftis G, Brown VA, Antonarakis SE. Recurrent mutations in haemophilia A give evidence for CpG mutation hotspots. Nature 1986;324:380-382.

342. Berg L-P, Wieland K, Millar DS, Kakkar VV, Cooper DN. Detection of a Novel Point Mutation causing Haemophilia A by PCR/Direct Sequencing of Ectopically Transcribed FVIII mRNA. British Journal of Haematology 1990:76(Suppl.1):29.

343. Naylor JA, Green PM, Montandon AJ, Rizza CR, Giannelli F. Detection of three novel mutations in two haemophilia A patients by rapid screening of whole essential region of factor VIII gene. Lancet 1991;337:635-639.

344. Millar DS, Green PJ, Zoll B, Kakkar VV, Cooper DN. Carrier detection in haemophilia A by direct analysis of factor VIII gene lesions. Human Genetics 1991;87(1):99-100.

345. Pecorara M, Casarino L, Mori PG, Morfini M, Mancuso G, Scrivano AM, Boeri E, Molinari AC, De BR, Ciavarella N, Bencivelli F, Ripa T, Barbujani G, Loi A, Perfseu L, Cao A, Pirastu M. Hemophilia A: carrier detection and prenatal diagnosis by DNA analysis. Blood 1987;70(2):531-535.

346. Chan V, Tong TMF, Chan TPT, Tang M, Wan CW, Chan FY, Chu YC, Chan TK. Multiple Xba I polymorphisms for carrier detection and prenatal diagnosis of haemophilia A. British Journal of Haematology 1989;73:497-500.

347. Wehnert M, Schröder, Herrmann FH. A new marker at DXS115 useful for carrier detection in hemophilia A. Human Genetics 1990;86:59-60.

348. Taylor SAM, Bridge PJ, Lillicrap DP. A BstX I polymorphism detected by the factor VIII genomic probe p482.6 (F8C). Nucleic Acids Research 1989;17:6426.

349. Patterson M, Gitschier J, Bloomfield J, Bell M, Dorkins H, Frosster-Iskenius U, Sommer S, Sobell J, Schaid D, Thibodeau S, Davies KE. An intronic region within the human factor VIII gene is duplicated within Xq28 and is homologous to the polymorphic locus DXS115 (767). American Journal of Human Genetics 1898;679-685.

350. Lillicrap D, Holden JJA, Giles AR, White BN. Carrier detection strategy in haemophilia A: the benefits of combined DNA marker analysis and coagulation testing in sporadic haemophilic families. British Journal of Haematology 1988;70:321-326.

351. Bowen DJ, Thomas P, Bignell P, Peake IR. Polymerase Chain Reaction (PCR) Amplification of Three Intragenic DNA Polymorphisms within the Factor IX Gene. British Journal of Haematology 1990:76(Suppl. 1):15.

352. Lillicrap DP, Liddell MB, Matthews RJ, Peake IR, Bloom AL. Comparison of phenotypic assessment and the use of two restriction fragment length polymorphisms in the diagnosis of the carrier state in haemophilia B. British Journal of Haematology 1986;62:557-565.

353. Winter RM, Harper K, Goldman E, Mibashan RS, Warren RC, Rodeck CH, Denketh RJA, Ward RHJ, Hardisty RM, Pembrey ME. First trimester prenatal diagnosis and detection of carriers of haemophilia A using the linked probe DX13. British Medical Journal 1985;291:765-769.

354. Peake IR, Lillicrap DP, Liddell MB, Matthews RJ, Bloom AL. Linked and intragenic probes for haemophilia A. Lancet 1985;ii:1003-1004.

355. Driscoll C, Miller CH, Goldberg JD, Aledort LM, Hoyer LW, Golbus MS. Recombination between the factor VIIIC gene and the ST14 locus. Lancet 1986;ii:279.

356. Lehesjoki AE, de la Chapelle A, Rasi V. Haemophilia A: two recombinations detected with the probe ST14. Lancet 1986;ii:280.

357. Haldane JBS. The rate of spontaneous mutation of a human gene. Journal of Genetics 1935;31:317-326.

358. Biggs R, Rizza CR. The sporadic case of haemophilia A. Lancet 1976;ii:431-433.

359. Grover H, Phillips MA, Lillicrap DP, Giles AR, Garvey MB, Teitel J, Rivard G, Blanchard V, White BN, Holden JJA. Carrier detection of haemophilia A using DNA markers in families with an isolated affected male. Clinical Genetics 1987;32:10-19.

360. Bernardi F, Marchetti G, Bertagnolo V, Faggioli L, Volinia S, Patracchini P, Bartolai S, Vannini F, Feloni L, Rossi L, Panicuci F, Conconi F. RFLP analysis in families with sporadic haemophilia A. Estimate of the mutation ratio in male and female gametes. Human Genetics 1987;76:253-256.

361. Vogel F, Rathenberg R. Spontaneous mutation in men. In: Harris H, Hirschorn K (eds.) Advances in Human Genetics. Plenum Press, New York, pp.223-318.

362. Antonarakis SW, Kazazian HH. Hemophilia A in man: molecular defects in the factor VIII gene. In: Hoffbrand AV (ed.) Recent Advances in Haematology, vol.5, Edinburgh, Churchill Livingstone. pp.243-250.

363. Higuchi M, Kochhan L, Olek K. A somatic mosaic for Haemophilia A detected at the DNA level. Molecular Biology in Medicine 1988;5:23-27.

364. Mazurier C, Gaucher C, Jorieux S, Parquet-Gernez A, Goudemand M. Evidence for a von Willebrand factor defect in factor VIII binding in three members of a family previously misdiagnosed mild haemophilia A and haemophilia A carriers: consequences for therapy and genetic counselling. British Journal of Haematology 1990;76:372-379.

Appendix 1.

Pedigree, Phenotypic and Genotypic Data on Families with Haemophilia A.

Key to Family Trees.

Normal female

Carrier female

Haemophilic female

Normal male

Haemophilic male

Deceased

Index member (or members)

N/A Not applicable

Roman numerals indicate generation number.

CD - Carrier detection

AND - Antenatal diagnosis

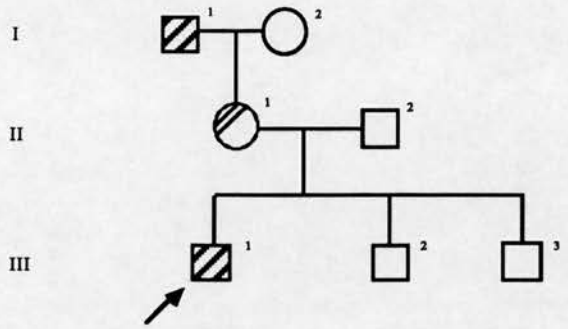
Restriction Fragment Length Polymorphisms.

Factor VIII Gene.	**Fragment sizes**
Bcl I	0.8/1.1kb
Xba I	4.8/6.2kb
Bgl I	5.0/20.0kb
Bgl II	2.8/5.8kb
Taq I	Multiple

Factor IX Gene.	
Taq I	1.3/1.8kb
Xmn I	6.5/11.5kb
Dde I	1.70/1.75kb

Family No. 1

Pedigree



Pedigree and Phenotype Data.

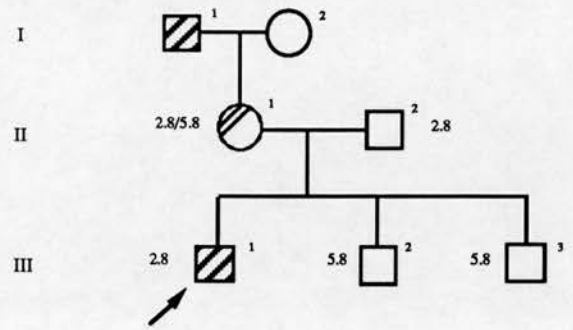
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-1	F	1.50	2.30	1.00	0.65	1.50	Obligate carrier	Normal
II-2	M	1.10	1.25	1.05	0.88	1.04		
III-1	M	0.02	0.72	1.00	-	-	Propositus	
III-2	M	0.80	0.70	1.00	1.14	0.80		
III-3	M	1.20	1.30	-	0.92	-		

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
II-1	F	0.8/0.8	5.0/5.0	6.2/6.2	5.8/2.8	N/A - Obligate carrier
II-2	M	0.8	5.0	6.2	2.8	
III-1	M	0.8	5.0	6.2	2.8	Propositus
III-2	M	0.8	5.0	6.2	5.8	
III-3	M	0.8	5.0	6.2	5.8	

Family No. 1 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

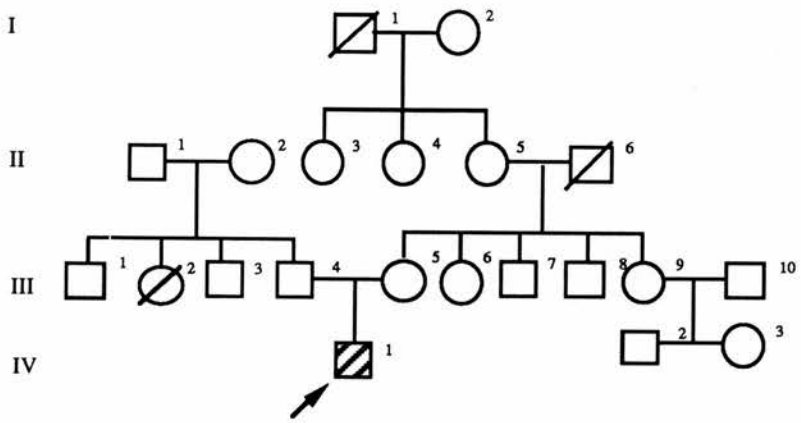
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-1	Obligate carrier	Normal	Informative Bgl II: Obligate carrier	N/A	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the 2.8kb Bgl II allele. II-1 is the daughter of a severely affected haemophiliac and is, therefore, an obligate carrier. She is informative for the linked Bgl II polymorphism which could be used for antenatal diagnosis but with a 5% chance of error due to possible recombination.

Family No. 2

Pedigree



Pedigree and Phenotype Data.

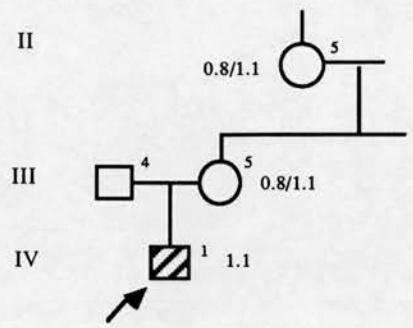
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-5	F	2.05	2.00	1.10	1.02	1.86	Potential carrier	Normal
III-5	F	1.35	1.15	1.10	1.17	1.23	Potential carrier	Normal
IV-1	M	0.01	1.10	-	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl II	Carrier status from Genotype
II-5	F	0.8/1.1	5.8/5.8	Potential carrier
III-5	F	0.8/1.1	5.8/5.8	Potential carrier
IV-1	M	1.1	5.8	Propositus

Family No. 2 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 3

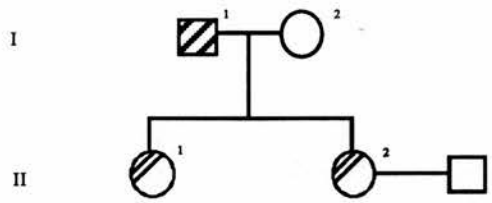
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-5	Potential carrier	Normal	Informative Bcl I: Potential carrier	Unhelpful	Yes
III-5	Potential carrier	Normal	Informative Bcl I: Potential carrier	Unhelpful	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the 1.1kb Bcl I allele. III-5 and II-5 cannot be excluded as carriers by either pedigree, phenotypic or genotypic analysis. Both are informative for the Bcl I polymorphism and could be offered prenatal diagnosis if requested. It is unclear if the mutation in IV-1 is unique or inherited. It is possible that by extending the pedigree data the origin of the abnormal gene could be predicted allowing a more accurate assessment of carrier status in the 'at-risk' women. However, within this limited pedigree ideally, the mutation in IV-1 should be identified and then used to clarify the carrier status of the 'at-risk' women.

Family No. 3

Pedigree



Pedigree and Phenotype Data.

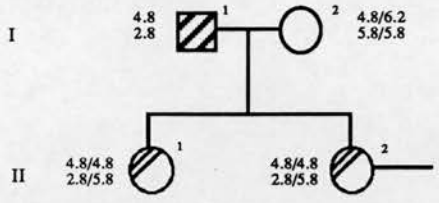
Gen. No.	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-1	M	0.00	-	-	-	-	Propositus	
I-2	F	-	-	-	-	-	Normal	No data
II-1	F	0.29	0.69	-	0.42	-	Obligate carrier	Carrier
II-2	F	1.07	1.02	-	1.05	-	Obligate carrier	Normal

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
I-1	M	0.8	5.0	4.8	2.8	Propositus
I-2	F	0.8/0.8	5.0/5.0	4.8/6.2	5.8/5.8	Normal
II-1	F	0.8/0.8	-	4.8/4.8	2.8/5.8	N/A - Obligate carrier
II-2	F	0.8/0.8	-	4.8/4.8	2.8/5.8	N/A - Obligate carrier

Family No. 3 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

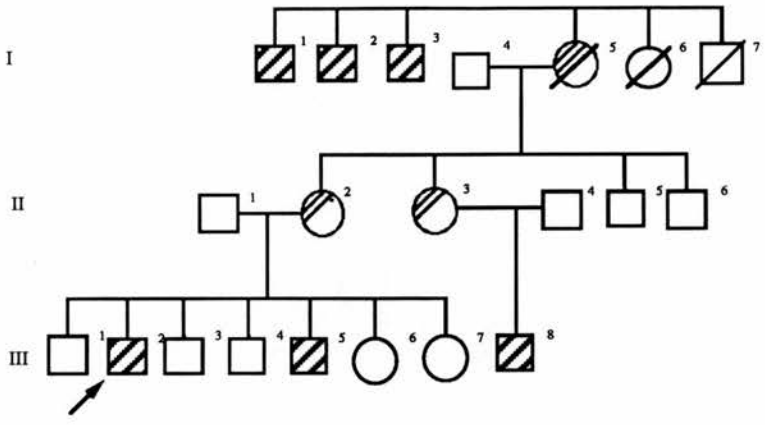
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-1	Obligate carrier	Carrier	Informative Bgl II: Obligate carrier	N/A	Yes
II-2	Obligate carrier	Normal	Informative Bgl II: Obligate carrier	N/A	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [4.8/2.8kb] Xba I/Bgl II haplotype.
II-1 and II-2 are both daughters of a severely affected haemophiliac and are, therefore, both obligatory carriers. Although phenotypic data supports II-1 as being a carrier it is normal in the case of II-2, emphasising the differences in Lyonisation which must have occurred. They are both informative for the linked Bgl II polymorphism which could be used for prenatal diagnosis although with a 5% chance of error because of possible recombination.

Family No. 4

Pedigree



Pedigree and Phenotype Data.

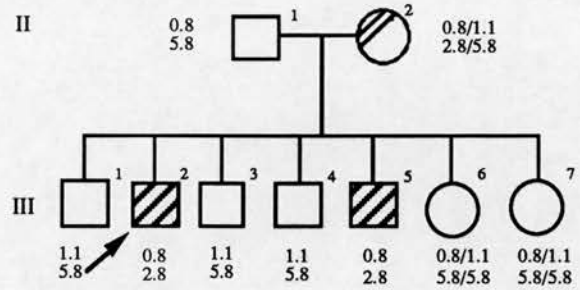
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-1	M	1.20	1.30	1.10	0.92	1.10		
II-2	F	1.00	1.25	0.96	0.80	1.04	Obligate carrier	Normal
III-1	M	1.00	0.82	0.36	1.22	2.70		
III-2	M	0.08	0.93	0.90	-	-	Propositus	
III-3	M	1.10	0.89	-	1.24	-		
III-4	M	1.05	0.70	1.30	1.50	0.81		
III-5	M	0.08	0.91	0.70	-	-	Propositus	
III-6	F	1.00	0.85	1.00	1.18	1.00	Potential carrier	Normal
III-7	F	0.80	0.75	0.35	1.07	2.29	Potential carrier	Normal

Genotype Data.

Gen. No	Sex	Bcl I	Bgl II	Carrier status from Genotype
II-1	M	0.8	5.8	
II-2	F	0.8/1.1	2.8/5.8	N/A - Obligate carrier
III-1	M	1.1	5.8	
III-2	M	0.8	2.8	Propositus
III-3	M	1.1	5.8	
III-4	M	1.1	5.8	
III-5	M	0.8	2.8	Propositus
III-6	F	0.8/1.1	5.8/5.8	Normal
III-7	F	0.8/1.1	5.8/5.8	Normal

Family No. 4 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 9

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic data	Applications of genotypic data to: CD	AND
II-2	Obligate carrier	Normal	Informative Bcl I/ Bgl II: Obligate carrier	N/A	Yes
III-6	Potential carrier	Normal	Informative Bcl I: Normal	Yes	N/A
III-7	Potential carrier	Normal	Informative Bcl I: Normal	Yes	N/A

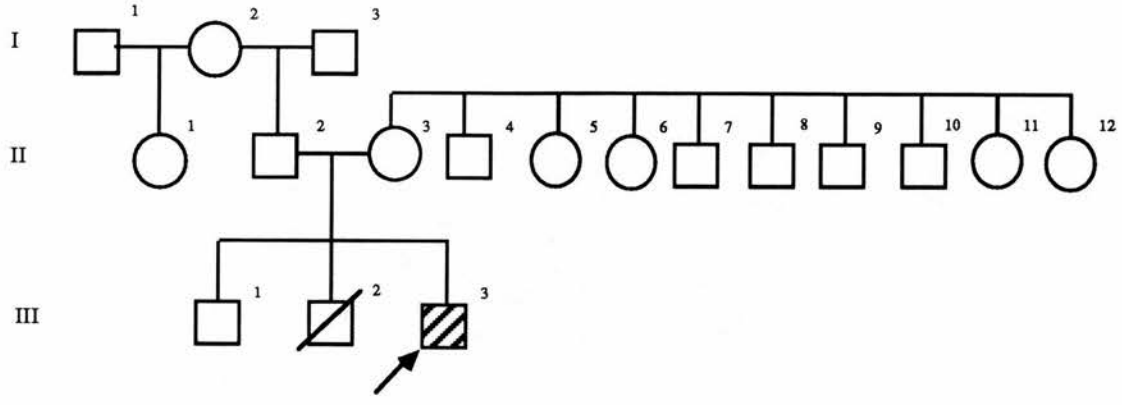
Implications and interpretations from these studies.

II-2 is an obligate carrier having two affected sons and a family history of haemophilia A. III-6 and III-7 are both potential carriers from the pedigree although their coagulation phenotypes are normal.

The haemophilic gene in this kindred is associated with the 0.8kb allele of the Bcl I polymorphism. III-6 and III-7 have inherited this allele from their father rather than their carrier mother and are, therefore, both normal.

Family No. 5

Pedigree



Pedigree and Phenotype Data.

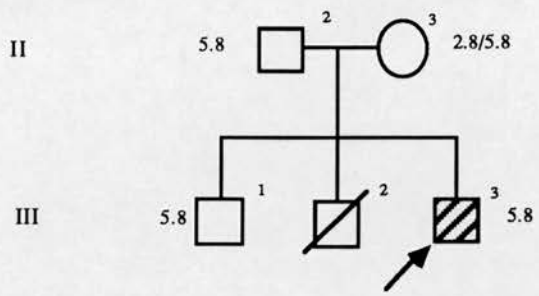
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-2	M	1.00	0.54	0.75	1.85	1.33		
II-3	F	0.64	0.75	1.00	0.85	0.64	Potential carrier	Normal
III-1	M	0.94	0.52	1.00	1.81	0.94		
III-3	M	0.02	1.00	0.75			Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Bgl II	Carrier status from Genotype
II-2	M	0.8	5.0	5.8	
II-3	F	0.8/0.8	5.0/5.0	2.8/5.8	Normal
III-1	M	0.8	5.0	5.8	
III-3	M	0.8	5.0	5.8	Propositus

Family No. 5 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

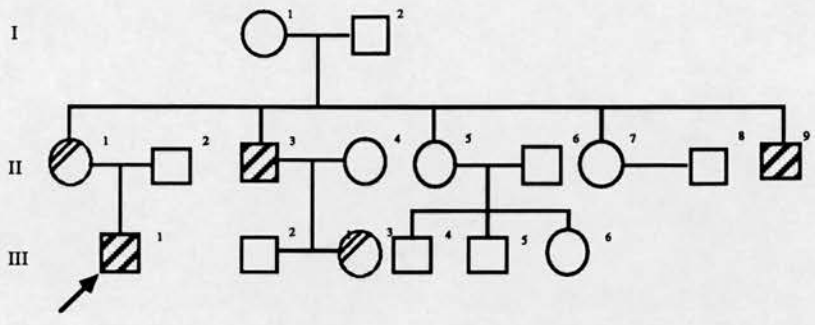
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-3	Potential carrier	Normal	Informative Bgl II: Normal	Yes	N/A

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is associated with the 5.8kb allele of the Bgl II polymorphism. II-3 is a potential carrier having a single affected son. The coagulation phenotype is normal. However, both III-1 and III-3 have the same haplotype indicating the mutation in III-3 is unique (as III-1 is phenotypically normal). II-3, unless a mosaic is, therefore, not a carrier.

Family No. 6

Pedigree



Pedigree and Phenotype Data.

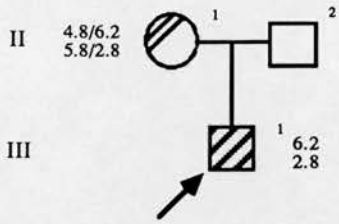
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-1	F	0.51	0.78	-	0.61	-	Obligate carrier	Carrier
III-1	M	0.07	1.10	0.56	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Xba I	Bgl II	Carrier status from Genotype
II-1	F	0.8/0.8	4.8/6.2	5.8/2.8	N/A - Obligate carrier
III-1	M	0.8	6.2	2.8	Propositus

Family No. 6 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 2

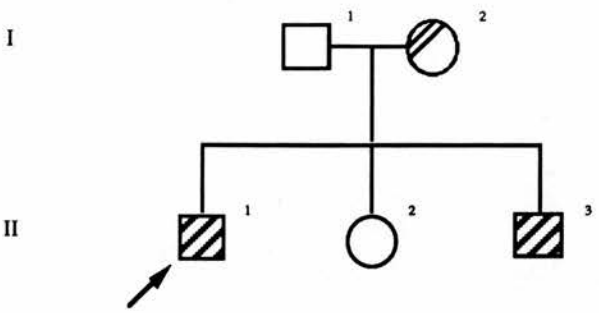
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-1	Obligate carrier	Carrier	Informative Xba I/ Bgl II: Obligate carrier	N/A	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [2.8/6.2kb] haplotype of the Xba I/Bgl II polymorphisms. II-1 is an obligate carrier having a single affected son and a family history of haemophilia. Her coagulation phenotype is abnormal. She is informative for both the Xba I and Bgl II polymorphisms and could be offered prenatal diagnosis if requested.

Family No. 7

Pedigree



Pedigree and Phenotype Data.

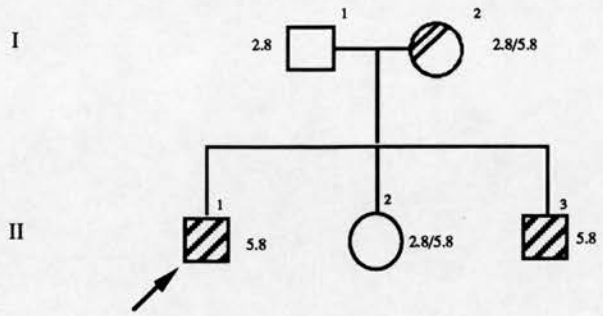
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-2	F	0.35	0.65	0.70	0.54	0.50	Obligate carrier	Carrier
II-1	M	0.00	1.25	0.32	-	-	Propositus	
II-2	F	0.48	0.62	-	0.78	-	Potential carrier	Carrier

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
I-1	M	1.1	5.0	6.2	2.8	
I-2	F	1.1/1.1	5.0/5.0	6.2/6.2	5.8/2.8	N/A - Obligate carrier
II-1	M	1.1	5.0	6.2	5.8	Propositus
II-2	F	1.1/1.1	5.0/5.0	6.2/6.2	5.8/2.8	Carrier
II-3	M	1.1	5.0	6.2	5.8	Propositus

Family No. 7 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Obligate carrier	Carrier	Informative Bgl II: Obligate carrier	N/A	Yes
II-2	Potential carrier	Carrier	Informative Bgl II: Carrier	Yes	Yes

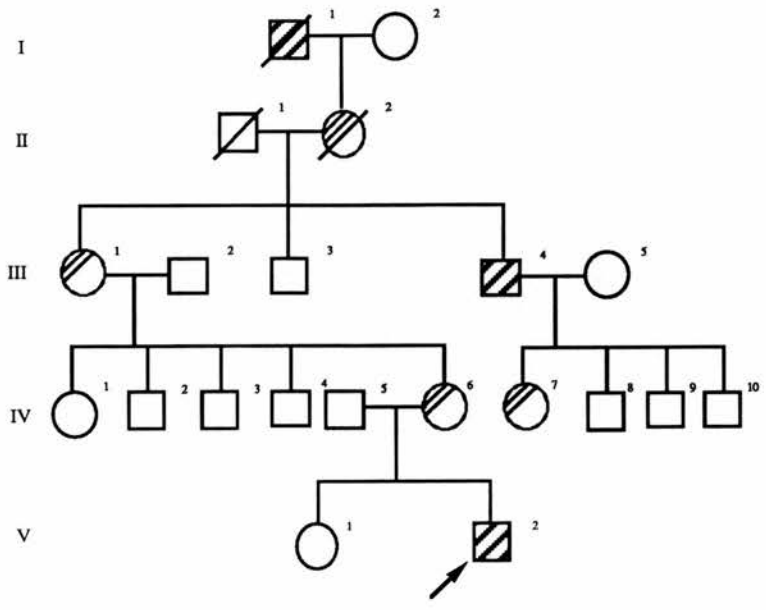
Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the 5.8kb Bgl II allele. I-2 is an obligate carrier having 2 affected sons and in addition has an abnormal coagulation phenotype. She is informative for only the linked Bgl II polymorphism but which could be used in prenatal diagnosis.

II-2 is a potential carrier from the pedigree and has a low VIII:C suggestive of carriership although a normal VIII:C/vWF:Ag ratio. Genotypic analysis shows she has inherited the abnormal 5.8kb BgI II allele from her carrier mother Both I-2 and II-2 are informative for the Bgl II polymorphism which could be used for prenatal diagnosis although with a 10% chance of error due to two possible recombinations each of 5%.

Family No. 8

Pedigree



Pedigree and Phenotype Data.

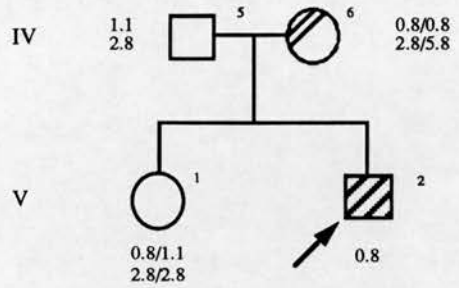
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
IV-5	M	0.90	0.48	1.15	1.88	0.78		
IV-6	F	0.95	0.76	0.84	1.25	1.13	Obligate carrier	Normal
V-1	F	0.95	1.00	0.61	0.95	1.56	Potential carrier	Normal
V-2	M	0.19	1.00	-	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
IV-5	M	1.1	-	-	2.8	
IV-6	F	0.8/0.8	20/20	4.8/4.8	2.8/5.8	N/A - Obligate carrier
V-1	F	0.8/1.1	-	-	2.8/2.8	Potential carrier
V-2	M	0.8	20	4.8	-	Propositus

Family No. 8 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
IV-6	Obligate carrier	Normal	Informative Bgl II: Obligate carrier	N/A	Yes
V-1	Potential carrier	Normal	Informative Bgl II/ Bcl I: Potential carrier	Yes	Yes

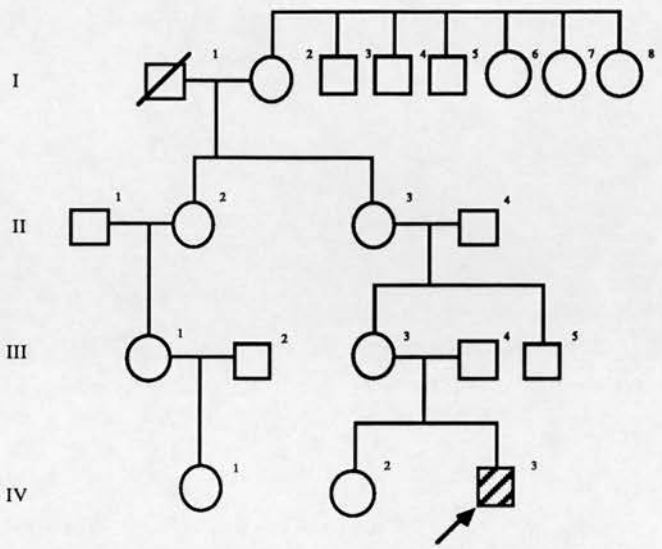
Implications and interpretations from these studies.

IV-6 is an obligate carrier having a single affected son and a family history of haemophilia although her coagulation phenotype is normal. She is informative for only the linked Bgl II polymorphism which could be used for prenatal diagnosis but with a 5% chance of error due to recombination.

V-1 is a potential carrier although phenotypically normal. Insufficient DNA was available on the propositus to perform Bgl II studies. The carrier status of V-1 cannot, therefore, be established.

Family No. 9

Pedigree



Pedigree and Phenotype Data.

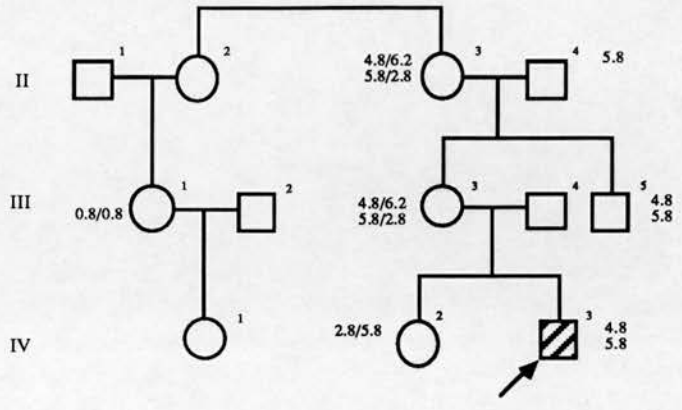
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-3	F	0.92	1.30	0.83	0.71	1.11	Potential carrier	Normal
II-4	M	No data					Clinically normal	
III-1	F	1.45	1.53	1.48	0.95	0.98	Potential carrier	Normal
III-3	F	0.20	0.68	1.00	0.29	0.20	Potential carrier	Carrier
III-4	M	1.00	1.52	-	1.52	-		
III-5	M	0.90	2.60	1.60	0.34	0.56		
IV-2	F	1.05	1.20	-	0.87	-	Potential carrier	Normal
IV-3	M	0.00	0.40	0.60	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
II-3	F	0.8/0.8	-	4.8/6.2	2.8/5.8	Normal
II-4	M	0.8	-	-	5.8	
III-1	F	0.8/0.8	-	-	-	Normal
III-3	F	0.8/0.8	5.0/5.0	4.8/6.2	2.8/5.8	Potential carrier
III-5	M	0.8	-	4.8	5.8	
IV-2	F	0.8/0.8	-	-	2.8/5.8	Potential carrier
IV-3	M	0.8	5.0	4.8	5.8	

Family No. 9 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 7

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-3	Potential carrier	Normal	Informative Xba I/ Bgl II: Normal	Yes	N/A
III-1	Potential carrier	Normal	Normal	Yes	N/A
III-3	Potential carrier	Carrier	Informative Xba I/ Bgl II: Obligate carrier	Yes	Yes
IV-2	Potential carrier	Normal	Informative Bgl II: Potential carrier	Yes	Yes

Implications and interpretations from these studies.

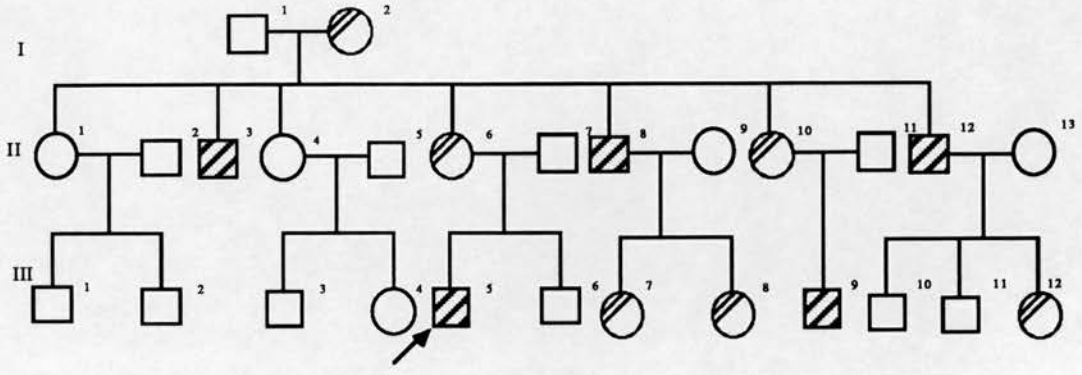
In this pedigree the haemophilic gene is marked by the [4.8/5.8kb] haplotype of the Xba I/Bgl II polymorphisms. III-5 has inherited the [4.8/5.8] haplotype from his mother - II-3 but is phenotypically normal. Therefore, the mutation must be unique to III-3 excluding II-2, III-1 and IV-1 as carriers.

III-3 has a carrier phenotype strongly suggesting she is a carrier. She is informative for both the Bgl II and Xba I polymorphisms and this has been used for prenatal diagnosis - an affected fetus was detected and the pregnancy terminated.

IV-2 is a potential carrier - unfortunately DNA was not available from her father and, therefore, genotypic analysis was not possible.

Family No. 10

Pedigree



Pedigree and Phenotype Data.

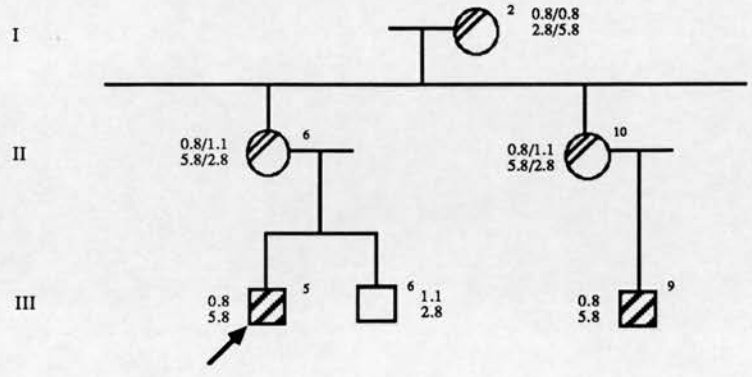
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-2	F	1.30	0.80	-	1.63	-	Obligate carrier	Normal
II-6	F	0.70	1.20	1.10	0.64	1.09	Obligate carrier	Normal
II-10	F	0.96	1.00	1.36	0.96	0.71	Obligate carrier	Normal
III-5	M	0.10	1.45	1.05	-	-		
III-6	M	1.00	1.00	0.74	1.00	1.35		
III-9	M	0.05	1.26	-	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl II	Carrier status from Genotype
I-2	F	0.8/0.8	2.8/5.8	N/A - Obligate carrier
II-6	F	0.8/1.1	2.8/5.8	N/A - Obligate carrier
II-10	F	0.8/1.1	2.8/5.8	N/A - Obligate carrier
III-5	M	0.8	5.8	
III-6	M	1.1	2.8	
III-9	M	0.8	5.8	Propositus

Family No. 10 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 6

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Obligate carrier	Normal	Informative Bgl II: Obligate carrier	N/A	Yes
II-6	Obligate carrier	Normal	Informative Bcl I/ Bgl II: Obligate carrier	N/A	Yes
II-10	Obligate carrier	Normal	Informative Bcl I/ Bgl II: Obligate carrier	N/A	Yes

Implications and interpretations from these studies.

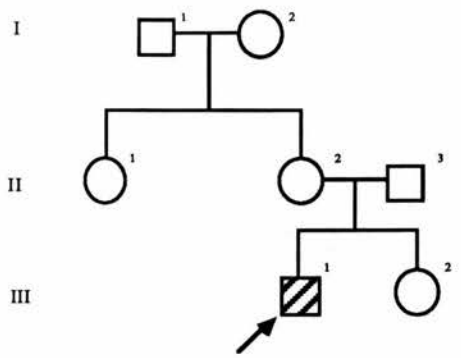
In this pedigree the haemophilic gene is marked by the [0.8/5.8kb] haplotype of the Bcl I/Bgl II polymorphisms. The family is unusual in that two of the affected individuals studied (III-5 and III-9) have differing VIII:C levels. II-6 and II-10 are both obligate carriers as each has a single affected son and there is a family history of haemophilia. Their mother (I-2) is also an obligate carrier having 3 affected sons. I-2, II-6 and II-10 all have normal coagulation phenotypes. They are informative for one or more polymorphisms and could be offered prenatal diagnosis if requested.

The explanation for the differing VIII:C values in the 2 haemophiliacs is unclear as presumably the underlying mutation is identical in each case.

Other members of this kindred were not available for study.

Family No. 11

Pedigree



Pedigree and Phenotype Data.

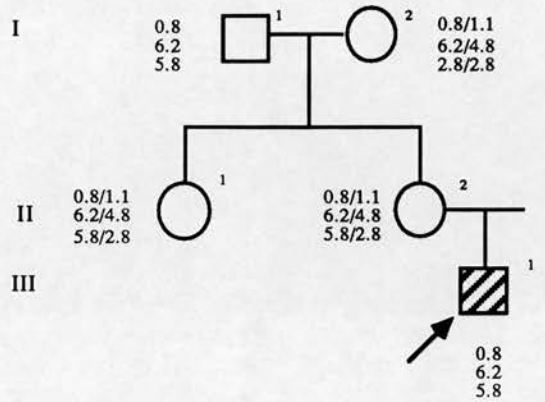
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-1	M	0.74	0.60	0.52	1.23	1.42		
I-2	F	0.84	0.80	0.27	1.05	3.11	Potential carrier	Normal
II-1	F	1.00	0.80	0.98	1.25	1.02	Potential carrier	Normal
II-2	F	0.22	0.84	0.30	0.26	0.73	Potential carrier	Carrier
III-1	M	0.00	0.64	0.89	-	-	Propositus	
III-2	F	1.10	0.72	0.87	1.52	1.26	Potential carrier	Normal

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
I-1	M	0.8	5.0	6.2	5.8	
I-2	F	0.8/1.1	5.0/5.0	4.8/6.2	2.8/2.8	Normal
II-1	F	0.8/1.1	5.0/5.0	4.8/6.2	2.8/5.8	Normal
II-2	F	0.8/1.1	5.0/5.0	4.8/6.2	2.8/5.8	Potential carrier
III-1	M	0.8	5.0	6.2	5.8	Propositus
III-2	No DNA sample available					

Family No. 11 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Potential carrier	Normal	Heterozygous Bcl I Xba I/Bgl II: Normal	Yes	N/A
II-1	Potential carrier	Normal	Heterozygous Bcl I/ Xba I/Bgl II: Potential carrier	Yes	N/A
II-2	Potential carrier	Carrier	Heterozygous Bcl I/ Xba I/Bgl II: Potential carrier	Yes	N/A
III-2	Potential carrier	Normal	No genotypic data available		

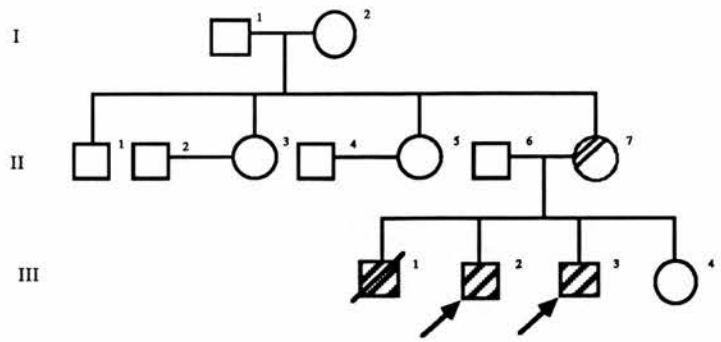
Implications and interpretations from these studies.

II-2 has an abnormal coagulation phenotype consistent with being a carrier. The haemophilic haplotype [0.8/6.2/5.8] is derived from I-1 who was clinically normally. Therefore, the mutation appears to be unique to II-2 excluding II-1 and I-2 as carriers. II-2 is informative for both the Xba I and Bcl I polymorphisms and could be offered prenatal diagnosis if requested.
DNA was not available on III-2 for carrier studies.

It is possible that I-1 is a germ-line mosaic and, therefore, that II-1 is at risk of being a carrier. Alternatively the mutation may be unique to II-2 excluding II-1 as a carrier. Ideally characterisation of the kindred specific mutation is required to allow accurate carrier detection.

Family No. 12

Pedigree



Pedigree and Phenotype Data.

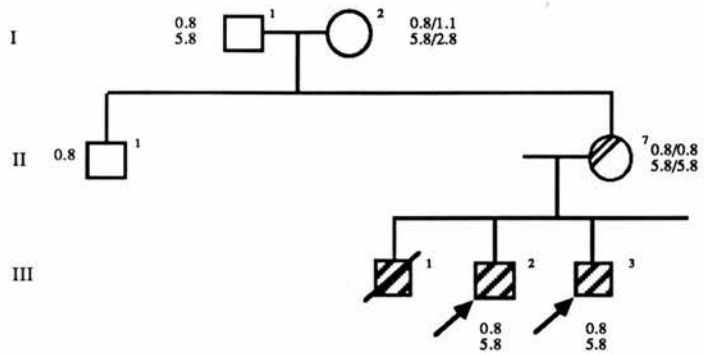
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-1	M	1.00	1.25	0.94	0.80	1.06		
I-2	F	1.00	1.00	0.80	1.00	1.25	Potential carrier	Normal
II-1	M	0.68	1.03	-	0.67	-		
II-7	F	0.50	1.35	1.00	0.38	0.37	Potential carrier	Carrier
III-2	M	0.00	1.23	1.90	-	-	Propositus	
III-3	M	0.00	1.25	0.76	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
I-1	M	0.8	5.0	-	5.8	
I-2	F	0.8/1.1	5.0/5.0	-	2.8/5.8	Normal
II-1	M	0.8	-	-	-	
II-7	F	0.8/0.8	5.0/5.0	6.2/6.2	5.8/5.8	N/A - Obligate carrier
III-2	M	0.8	5.0	6.2	5.8	Propositus
III-3	M	0.8	5.0	6.2	5.8	Propositus

Family No. 12 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 6

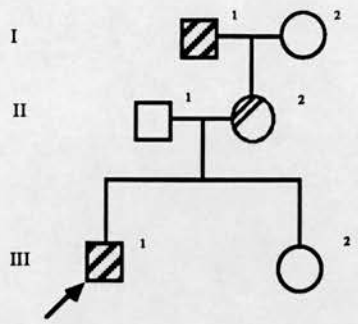
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Potential carrier	Normal	Informative Bcl I: Normal	N/A	-
II-7	Obligate carrier	Carrier	Non-informative: Obligate carrier	N/A	No

Implications and interpretations from these studies.

II-7 is an obligate carrier having 3 affected sons - her coagulation phenotype is also abnormal. It is not clear whether the haemophilic haplotype [0.8/5.8] is derived from the maternal grandfather or grandmother, although the finding of a 0.8kb allele in II-1 (phenotypically normal) suggests that I-2 is not a carrier and that the mutation has originated with I-1 (maternal grandfather). This is important in the genetic counselling of other potential carriers within the pedigree as I-1 may be a mosaic. Ideally, identification of the underlying defect in III-2 or III-3 is required which could then be used to establish the carrier status of the 'at-risk' women.

Family No. 13

Pedigree



Pedigree and Phenotype Data.

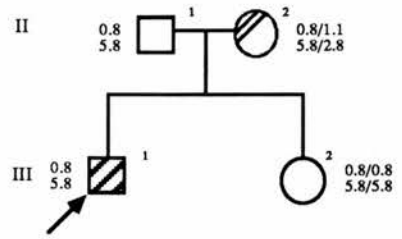
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-1	M	1.00	0.78	0.50	1.28	2.00		
II-2	F	0.46	0.68	0.62	0.68	0.74	Obligate carrier	Carrier
III-1	M	0.01	0.62	1.00	0.02	0.01	Propositus	
III-2	F	-	-	-	-	-	Potential carrier	No Data

Genotype Data.

Gen. No	Sex	Bcl I	Bgl II	Carrier status from Genotype
II-1	M	0.8	5.8	
II-2	F	0.8/1.1	2.8/5.8	N/A - Obligate carrier
III-1	M	0.8	5.8	Propositus
III-2	F	0.8/0.8	5.8/5.8	Carrier

Family No. 13 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

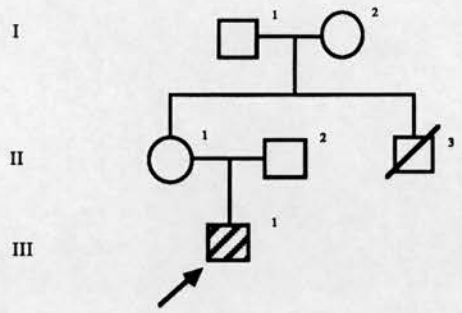
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-2	Obligate carrier	Carrier	Informative Bcl I/ Bgl II: Obligate carrier	N/A	Yes
III-2	Potential carrier	No Data	Non-informative: Carrier	Yes	No

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [0.8/5.8kb] Bcl I/Bgl II haplotype. II-2 is an obligate carrier as she is the daughter of a severely affected haemophiliac. She is informative for the Bcl I and Bgl II polymorphisms and can, therefore, be offered prenatal diagnosis if requested. III-2 is a potential carrier from the pedigree although no phenotypic data was available. She has inherited a 0.8kb allele from her father and the other 0.8kb allele from her mother and is, therefore, a carrier.
Insufficient DNA was available to study the other RFLP's in III-2 for use in prenatal diagnosis.

Family No. 14

Pedigree



Pedigree and Phenotype Data.

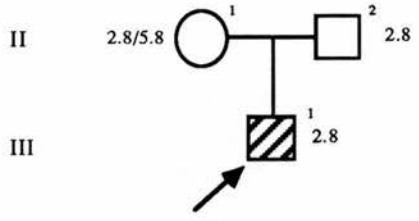
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-1	F	0.85	0.85	0.60	0.94	1.42	Potential carrier	Normal
II-2	M	1.00	0.90	1.00	1.11	1.00		
III-1	M	0.00	1.58	-	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
II-1	F	0.8/0.8	5.0/5.0	4.8/4.8	2.8/5.8	Potential carrier
II-2	M	0.8	5.0	4.8	2.8	Normal
III-1	M	0.8	5.0	4.8	2.8	Propositus

Family No. 14 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 3

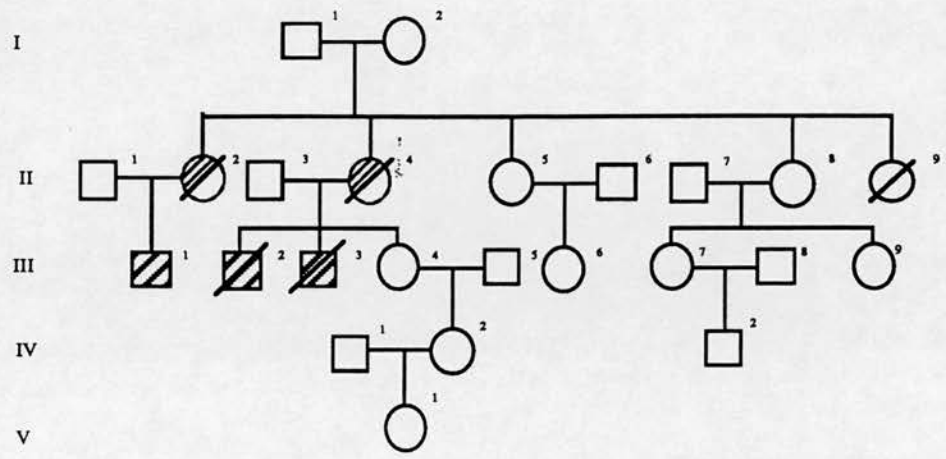
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-1	Potential carrier	Normal	Heterozygous Bgl II: Potential carrier	No	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the 2.8kb allele of the Bgl II polymorphism. II-1 is a potential carrier but has a normal phenotype. Although heterozygous for the Bgl II polymorphism her carrier status cannot be established. Ideally a kindred specific defect should be identified to more accurately determine if II-12 is a carrier or whether the mutation is unique to III-1.

Family No. 15

Pedigree



Pedigree and Phenotype Data.

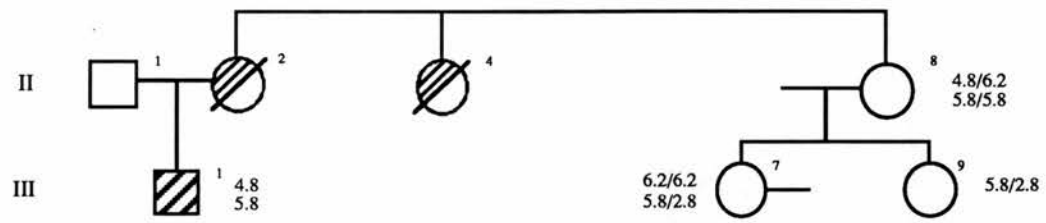
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-8	F	0.32	0.47	0.49	0.68	0.65	Potential carrier	Carrier
III-1	M	0.00	-	-	-	-	Propositus	
III-7	F	0.52	0.78	-	0.67	-	Potential carrier	Normal
III-9	F	0.16	1.02	0.88	0.16	0.18	Potential carrier	Carrier

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
II-8	F	0.8/0.8	5.0/5.0	4.8/6.2	5.8/5.8	Potential carrier
III-1	M	0.8	5.0	4.8	5.8	Propositus
III-7	F	0.8/0.8	5.0/5.0	6.2/6.2	5.8/2.8	Normal
III-9	F	0.8/0.8	5.0/5.0		2.8/5.8	Potential carrier

Family No. 15 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

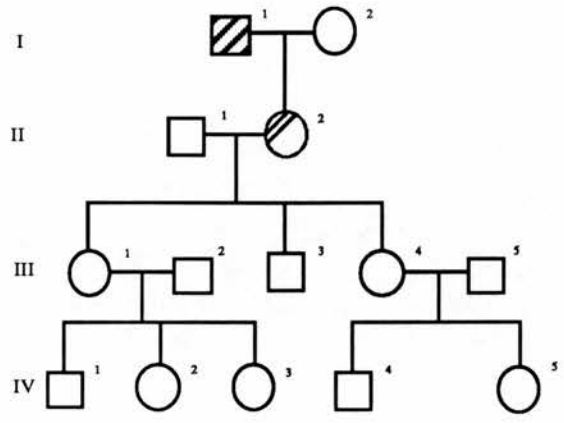
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-8	Potential carrier	Carrier	Informative Xba I: Potential carrier	Yes	Yes
III-7	Potential carrier	Normal	Informative Bgl II: Normal	Yes	N/A
III-9	Potential carrier	Carrier	Heterozygous Bgl II: Potential carrier	No	Yes

Implications and interpretations from these studies.

The haemophilic gene in this family is associated with the [4.8/5.8kb] Xba I/Bgl II haplotype. II-8 has a low VIII:C suggesting she is a carrier and genotypic analysis indicates she has inherited the [4.8/5.8kb] haplotype. Assuming this haplotype in II-8 indicates she is a carrier then III-7 has not inherited this haplotype and is, therefore, not a carrier. This is supported by a normal coagulation phenotype. Xba I analyses on III-9 were unsatisfactory and repeat samples were not available. However, she has both a low VIII:C and VIII:C/vWF:Ag ratio suggesting carriership.

Family No. 16

Pedigree



Pedigree and Phenotype Data.

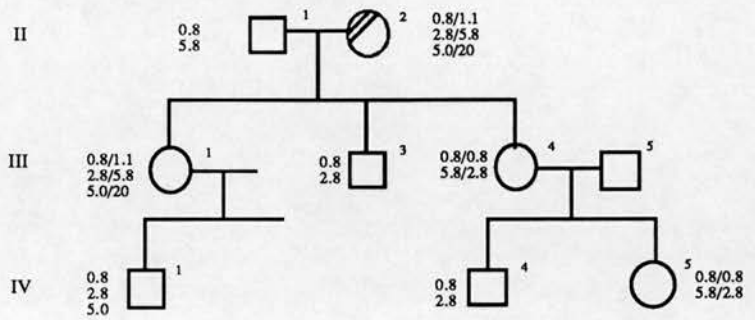
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from Pedigree	Phenotype
II-1	M	0.56	0.82	-	0.68	-	Normal	
II-2	F	0.27	1.65	1.65	0.16	0.16	Obligate carrier	Carrier
III-1	F	0.53	1.56	-	0.34	-	Potential carrier	Carrier
III-3	M	1.18	1.14	1.53	1.04	1.77	Normal	
III-4	F	0.69	0.63	0.63	1.10	1.10	Potential carrier	Normal
IV-1	M	1.60	1.50	1.04	1.06	1.54	Normal	
IV-4	M	0.82	0.51	-	1.61	-	Normal	
IV-5	F	0.65	0.90	-	0.72	-	Potential carrier	Normal

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
II-1	M	0.8	5.0	-	-	
II-2	F	0.8/1.1	-	-	2.8/5.8	N/A - Obligate carrier
III-1	F	0.8/1.1	5.0/20	6.2/6.2	2.8/5.8	Carrier
III-3	M	0.8	5.0	-	2.8	
III-4	F	0.8/0.8	-	-	2.8/5.8	Normal
IV-1	M	0.8	5.0	6.2	2.8	
IV-4	M	0.8	-	-	2.8	
IV-5	F	0.8/1.1	-	-	2.8/5.8	Normal

Family No. 16 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 8

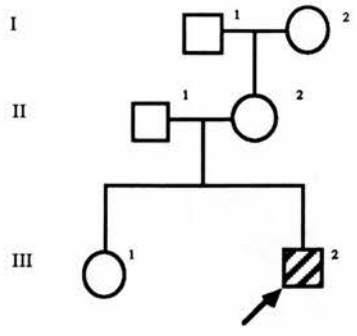
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-2	Obligate carrier	Carrier	Informative Bcl I/ Bgl II/Bgl I: Carrier	N/A	Yes
III-1	Potential carrier	Carrier	Informative Bcl I/ Bgl II/Bgl I: Carrier	Carrier	Yes
III-4	Potential carrier	Normal	Heterozygous Bgl II: Normal	Normal	N/A
IV-5	Potential carrier	Normal	Heterozygous Bgl II: Normal	Normal	N/A

Implications and interpretations from these studies.

In this kindred, no index member was available. However, II-2 is an obligate carrier as she is the daughter of a haemophiliac. Her son - III-3 is phenotypically normal and genotype analysis shows him to have inherited the [0.8/2.8/5.0kb] Bcl I/Bgl II/Bgl I haplotype. Therefore, the haemophilic gene in this kindred is associated with the other maternal haplotype [1.1/5.8/20.0kb].
From Bcl I and Bgl I analyses, III-1 has inherited the haemophilic haplotype (supported by an abnormal phenotype) whilst III-4 has inherited the normal haplotype (normal coagulation phenotype) excluding both herself and her daughter - IV-5 as carriers. However, using the linked Bgl II polymorphism shows that both III-1 and III-4 have inherited the normal 2.8kb Bgl II allele excluding them as carriers. This family illustrates one of the problems associated with the use of linked RFLP analyses - a recombination (cross-over) has taken place between II-2 and III-1. Non-paternity to explain these findings has not been excluded.
Antenatal diagnosis can be offered to III-1 using either the Bcl I or Bgl I polymorphisms.

Family No. 17

Pedigree



Pedigree and Phenotype Data.

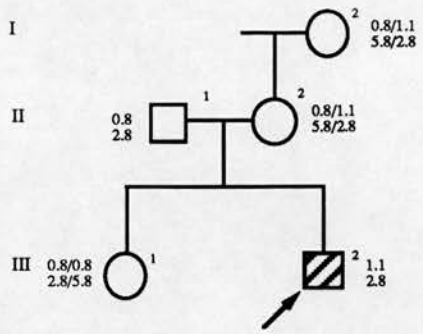
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-2	F	0.70	0.71	1.65	0.99	0.42	Potential carrier	Normal
II-2	F	0.86	1.38	1.60	0.62	0.53	Potential carrier	Normal
II-1	M	0.83	1.00	0.78	-	-		
III-1	F	0.52	0.46	0.86	1.13	0.60	Potential carrier	Normal
III-2	M	0.01	1.08	1.12	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl II	Carrier status from Genotype
I-2	F	0.8/1.1	2.8/5.8	Potential carrier
II-2	F	0.8/1.1	2.8/5.8	Potential carrier
II-1	M	0.8	2.8	
III-1	F	0.8/0.8	2.8/5.8	Normal
III-2	M	1.1	2.8	Propositus

Family No. 17 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Potential carrier	Normal	Heterozygous Bcl I/ Bgl II: Potential carrier	Yes	Yes
II-2	Potential carrier	Normal	Heterozygous Bcl I/ Bgl II: Potential carrier	No	Yes
III-1	Potential carrier	Normal	Normal	Yes	N/A

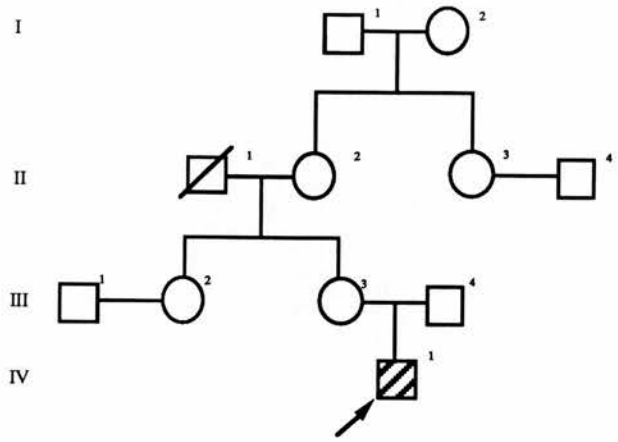
Implications and interpretations from these studies.

II-2 is a potential carrier with a normal coagulation phenotype. Is it unclear whether the haemophilic haplotype [1.1/2.8] is inherited from the maternal grandfather or grandmother. However, III-1 has inherited the non-haemophilic haplotype from her mother and is, therefore, normal.

This family emphasises that although it may be impossible to clarify the carrier status of some potential carriers, genotypic analysis can confidently exclude carriership in others.

Family No. 18

Pedigree



Pedigree and Phenotype Data.

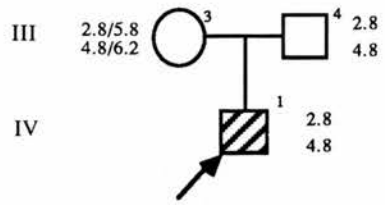
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
III-3	F	0.72	0.52	0.50	1.38	1.44	Potential carrier	Normal
III-4	M	1.25	1.90	0.90	0.66	1.39		
IV-1	M	0.02	1.24	0.82	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
III-3	F	0.8/0.8	5.0/5.0	4.8/6.2	2.8/5.8	Potential carrier
III-4	M	-	5.0	4.8	2.8	
IV-1	M	0.8	5.0	4.8	2.8	Propositus

Family No. 18 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 3

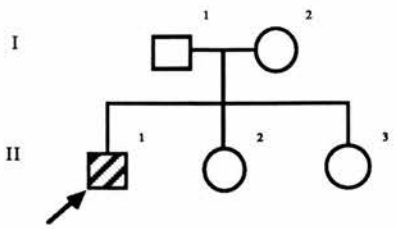
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
III-3	Potential carrier	Normal	Heterozygous Xba I/ Bgl II: Potential carrier	N/A	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [2.8/4.8kb] haplotype of the Xba I/Bgl II polymorphisms. III-3 is a potential carrier from the pedigree but has a normal coagulation phenotype. She is informative for the Xba I and Bgl II polymorphisms which could be used for prenatal diagnosis although these do not establish her carrier status. Ideally the mutation in the FVIII gene of IV-1 should be characterised and used for carrier detection studies.

Family No. 19

Pedigree



Pedigree and Phenotype Data.

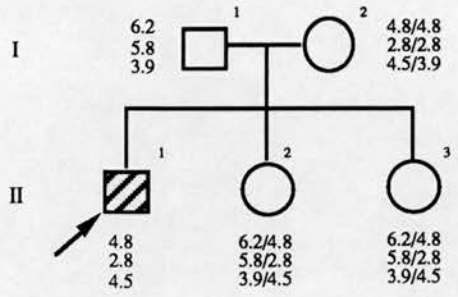
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-2	F	0.46	0.62	1.06	0.74	0.43	Potential carrier	Carrier
II-1	M	0.00	0.74	1.24	-	-	Propositus	
II-2	F	0.54	1.28	0.74	0.42	0.73	Potential carrier	Carrier
II-3	F	0.46	1.00	0.46	0.46	1.00	Potential carrier	Carrier

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Taq I	Carrier status from Genotype
I-1	M	0.8	5.0	6.2	5.8	3.9	
I-2	F	0.8/0.8	5.0/5.0	4.8/4.8	2.8/2.8	3.9/4.5	Potential carrier
II-1	M	0.8	5.0	4.8	2.8	4.5	Propositus
II-2	F	0.8/0.8	5.0/5.0	4.8/6.2	2.8/5.8	3.9/4.5	Potential carrier
II-3	F	0.8/0.8	5.0/5.0	4.8/6.2	2.8/5.8	3.9/4.5	Potential carrier

Family No. 19 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data Data/Status	Applications of genotypic data to: CD	AND
I-2	Potential carrier	Carrier	Informative Taq I: Potential carrier	Unhelpful	Yes
II-2	Potential carrier	Carrier	Informative Taq I: Carrier	Yes	Yes
II-3	Potential carrier	Carrier	Informative Taq I: Carrier	Yes	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [4.8/2.8/4.5kb] haplotype of the Xba I/ Bgl II/Taq I polymorphisms.

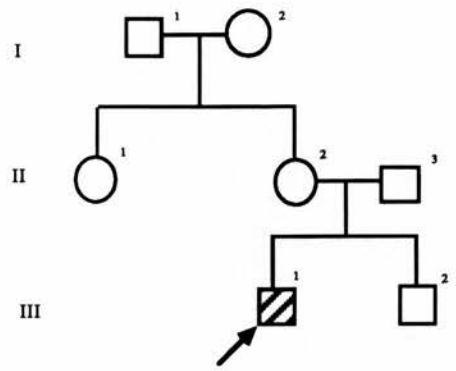
I-2, II-2 and II-3 are all potential carriers and all have abnormal coagulation phenotypes. The carrier status of I-2 cannot be definitely established by RFLP analysis, however, she has an abnormal coagulation phenotype suggestive of carriership.

Assuming I-2 is a carrier, then both II-2 and II-3 have inherited the haemophilic haplotype and are, therefore, carriers. This is supported by the finding of abnormal coagulation phenotypes in both these individuals. The finding of a carrier phenotype in both II-2 and II-3 (both with the haemophilic haplotype] reinforces the suggestion that I-2 is a carrier.

Both II-2 and II-3 are informative for the Xba I polymorphism which could be used for prenatal diagnosis. However, I-2 is informative for only the linked Taq I polymorphism and although this could be used for prenatal diagnosis there would be a risk of error due to possible recombination.

Family No. 20

Pedigree



Pedigree and Phenotype Data.

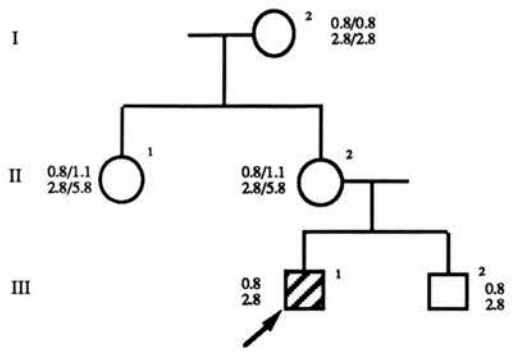
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-2	F	1.10	2.30	1.00	0.48	1.10	Potential carrier	Carrier
II-1	F	1.20	1.35	-	0.89	-	Potential carrier	Normal
II-2	F	1.00	2.00	1.70	0.50	0.58	Potential carrier	Carrier
III-1	M	0.00	1.00	-	-	-	Propositus	
III-2	M	1.30	0.90	0.66	1.4	2.1		

Genotype Data.

Gen. No	Sex	Bcl I	Bgl II	Carrier status from Genotype
I-2	F	0.8/0.8	2.8/2.8	Normal
II-1	F	0.8/1.1	2.8/5.8	Normal
II-2	F	0.8/1.1	2.8/5.8	Normal
III-1	M	0.8	2.8	Propositus
III-2	M	0.8	2.8	

Family No. 20 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

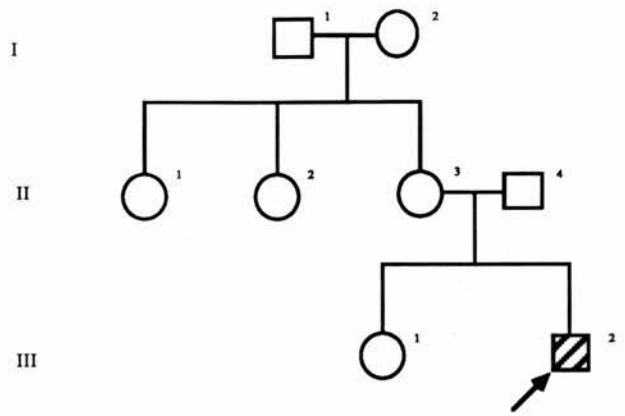
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Potential carrier	Normal	Normal	Yes	N/A
II-1	Potential carrier	Normal	Heterozygous Bcl I/ Xba I: Normal	Yes	N/A
II-2	Potential carrier	Normal	Heterozygous Bcl I/ Xba I: Normal	Yes	N/A

Implications and interpretations from these studies.

In the pedigree the haemophilic gene is marked by the [0.8/2.8kb] haplotype of the Bcl I/Bgl II polymorphisms.
I-2, II-1 and II-2 are all potential carriers and although their VIII:C values are normal, I-2 and II-2 have low VIII:C/vWF:Ag ratios. However, genotypic data shows that III-1 and III-2 have inherited the same haplotype [0.8/2.8] from their mother. As III-2 is phenotypically normal, this indicates that the mutation is unique to III-1 and therefore, the carrier status of the three potential carriers is established as normal

Family No. 21

Pedigree



Pedigree and Phenotype Data.

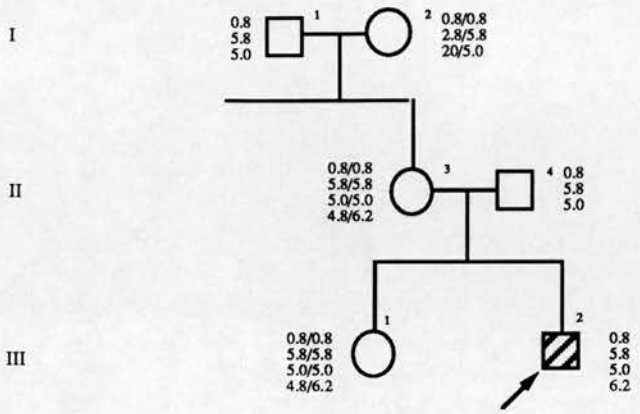
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-1	M	No coagulation data available						
I-2	F	1.00	1.85	1.08	0.54	0.92	Potential carrier	Carrier
II-3	F	0.68	0.99	1.40	0.69	0.48	Potential carrier	Carrier
II-4	M	0.74	1.02	0.94	0.73	0.79		
III-1	F	0.17	0.62	0.62	0.27	0.27	Potential carrier	Carrier
III-2	M	0.02	0.94	-	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
I-1	M	0.8	5.0	-	5.8	
I-2	F	0.8/0.8		-	2.8/5.8	Potential carrier
II-3	F	0.8/0.8	5.0/5.0	4.8/6.2	5.8/5.8	Potential carrier
II-4	M	0.8	5.0	4.8	5.8	
III-1	F	0.8/0.8	5.0/5.0	-	5.8/5.8	Potential carrier
III-2	M	0.8	5.0	6.2	5.8	Propositus

Family No. 21 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 6

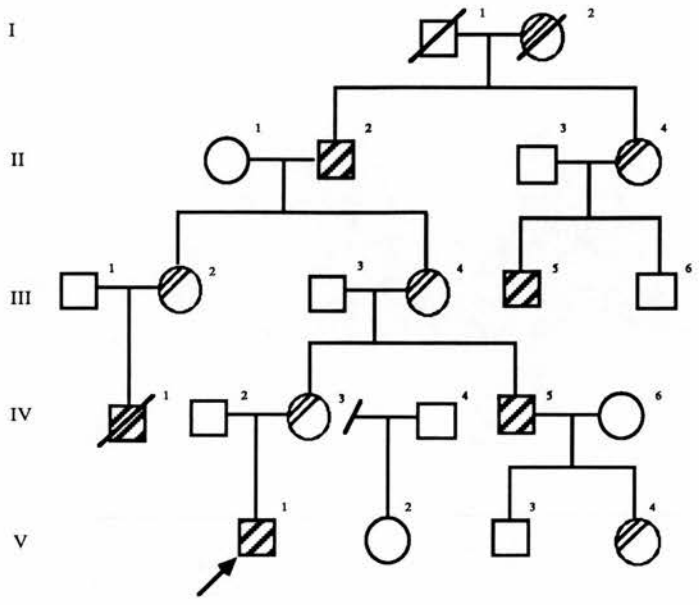
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Potential carrier	Carrier	Heterozygous Bgl I: Potential carrier	Unhelpful	Yes
II-3	Potential carrier	Carrier	Heterozygous Xba I: Potential carrier	Unhelpful	Yes
III-1	Potential carrier	Carrier	Heterozygous Xba I: Potential carrier	Unhelpful	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [0.8/5.8/5.0/6.2] Bcl I/Bgl II/Bgl I/Xba I haplotype. However, the origin of this haplotype is unclear as Xba I data was available only on limited family members. The finding of an abnormal coagulation phenotype in all 3 'at-risk' females suggests they may be carriers. If II-3 is a carrier, then III-1 has inherited the haemophilic haplotype suggesting carriership. II-3 and III-2 are informative for the intragenic Xba I polymorphism and I-2 for the Bgl I RFLP - all three, therefore, could be offered prenatal diagnosis if requested.

Family No. 22

Pedigree.



Pedigree and Phenotype Data.

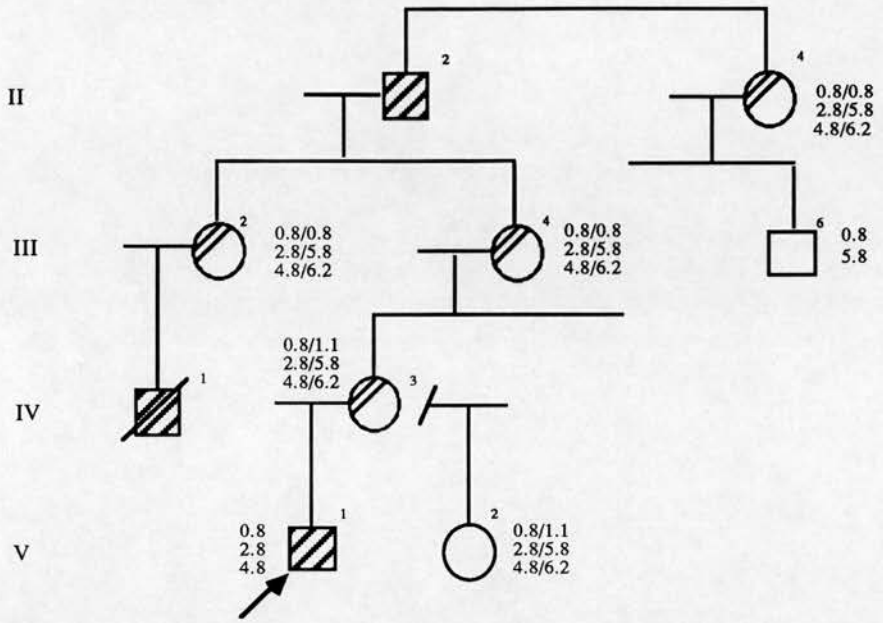
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-4	F	0.50	1.10	0.85	0.45	0.59	Obligate carrier	Carrier
III-2	F	0.27	0.60	0.88	0.45	0.31	Obligate carrier	Carrier
III-4	F	0.33	1.00	0.89	0.33	0.37	Obligate carrier	Carrier
III-6	M	0.61	0.58	0.72	1.05	0.85		
IV-3	F	0.18	0.56	0.41	0.32	0.44	Obligate carrier	Carrier
V-1	M	0.00	1.20	0.50	-	-	Propositus	
V-2	F	0.41	0.62	0.61	0.66	0.67	Potential carrier	Carrier

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
II-4	F	0.8/0.8	5.0/5.0	4.8/6.2	2.8/5.8	N/A - Obligate carrier
III-2	F	0.8/0.8	5.0/5.0	4.8/6.2	2.8/5.8	N/A - Obligate carrier
III-4	F	0.8/0.8	5.0/5.0	4.8/6.2	2.8/5.8	N/A - Obligate carrier
III-6	M	0.8	5.0	-	5.8	
IV-3	F	0.8/1.1	5.0/5.0	4.8/6.2	2.8/5.8	N/A - Obligate carrier
V-1	M	0.8	5.0	-	2.8	Propositus
V-2	F	0.8/0.8	5.0/5.0	4.8/6.2	2.8/5.8	Potential carrier

Family No. 22 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 7

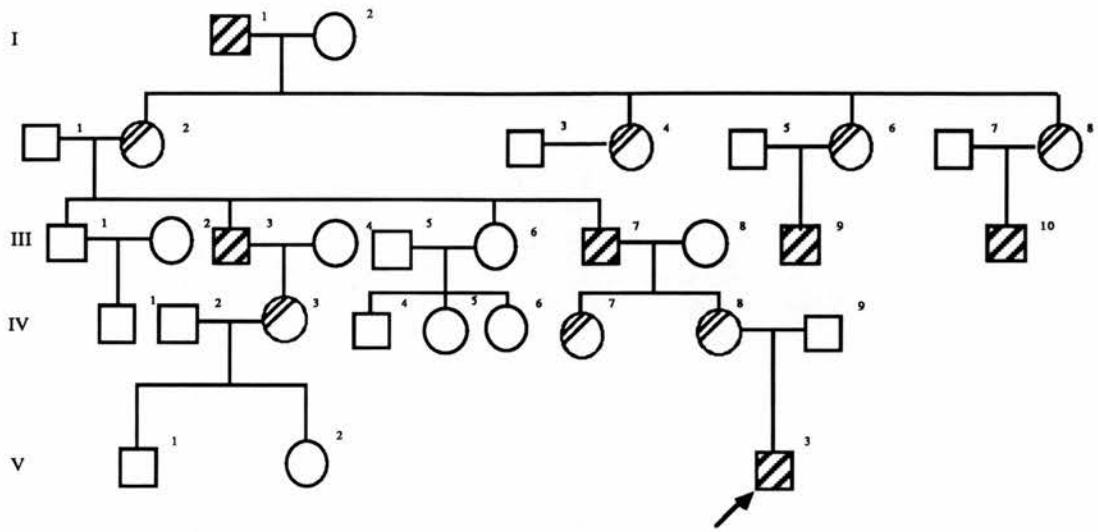
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-4	Obligate carrier	Carrier	Informative Xba I/ Bgl II: Obligate carrier	N/A	Yes
III-2	Obligate carrier	Carrier	Informative Xba I/ Bgl II: Obligate carrier	N/A	Yes
III-4	Obligate carrier	Carrier	Informative Xba I/ Bgl II: Obligate carrier	N/A	Yes
IV-3	Obligate carrier	Carrier	Informative Xba I/ Bcl I, Bgl II: Obligate carrier	N/A	Yes
V-2	Potential carrier	Carrier	Heterozygous Xba I/ Bcl I, Bgl II:Potential carrier	Yes	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [0.8/2.8/4.8kb] Bcl I/Bgl II/Xba I haplotype. III-2, III-4 and IV-3 are all obligate carriers and have abnormal coagulation phenotypes. They are all informative for one or more intragenic polymorphisms and could be offered prenatal diagnosis if requested. V-2 is a potential carrier but a DNA sample was not available from her biological father to permit carrier detection.

Family No. 23

Pedigree.



Pedigree and Phenotype Data.

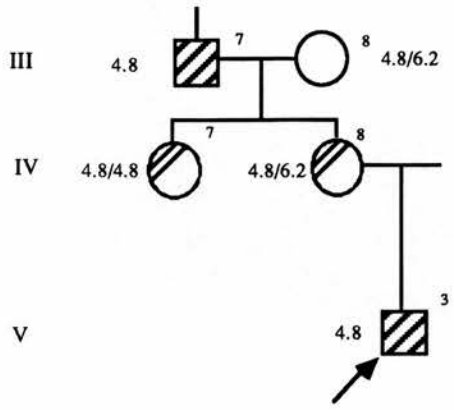
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
III-7	M	0.11	1.28	0.82	0.09	0.13		
III-8	F	1.00	0.62	0.84	1.61	1.12	Normal	Normal
IV-7	F	0.35	0.80	-	0.44	-	Obligate carrier	Carrier
IV-8	F	0.19	0.43	0.56	0.44	0.34	Obligate carrier	Carrier
V-3	M	0.14	0.40	0.59	-	-	Propositus	

Genotype Data.

Gen.	Sex No	Bcl I	Xba I	Bgl I	Bgl II	Carrier status from Genotype
III-7	M	0.8	4.8	5.0	5.8	
III-8	F	0.8/0.8	4.8/6.2	5.0/5.0	5.8/5.8	Normal
IV-7	F	0.8/0.8	4.8/4.8	5.0/5.0	5.8/5.8	N/A - Obligate carrier
IV-8	F	0.8/0.8	4.8/6.2	5.0/5.0	5.8/5.8	N/A - Obligate carrier
V-3	M	0.8	4.8	5.0	5.8	Propositus

Family No. 23 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

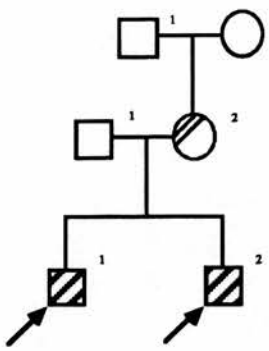
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
III-8	Normal	N/A	N/A	N/A	N/A
IV-7	Obligate carrier	Carrier	Non-informative: Obligate carrier	N/A	No
IV-8	Obligate carrier	Carrier	Informative Xba I: Obligate carrier	N/A	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [4.8kb] Xba I RFLP. IV-7 and IV-8 are both obligate carriers being the daughters of a severe haemophiliac. IV-8 is informative for the Xba I polymorphism which could be used in prenatal diagnosis. However, IV-7 is non-informative and insufficient DNA was available to analyse the Taq I polymorphism.

Family No. 24

Pedigree.



Pedigree and Phenotype Data.

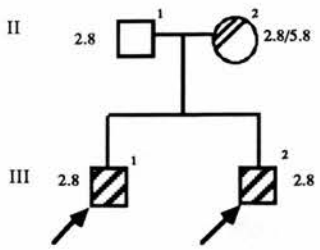
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-1	M	1.40	0.65	0.70	2.15	2.00		
II-2	F	0.52	1.35	2.00	0.39	0.26	Obligate carrier	Carrier
III-1	M	0.07	1.17	1.17	-	-	Propositus	
III-2	M	0.04	0.87	0.80	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
II-1	M	-	5.0	4.8	2.8	
II-2	F	0.8/0.8	5.0/5.0	4.8/4.8	2.8/5.8	N/A - Obligate carrier
III-1	M	0.8	5.0	4.8	2.8	
III-2	M	0.8	5.0	4.8	2.8	

Family No. 24 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

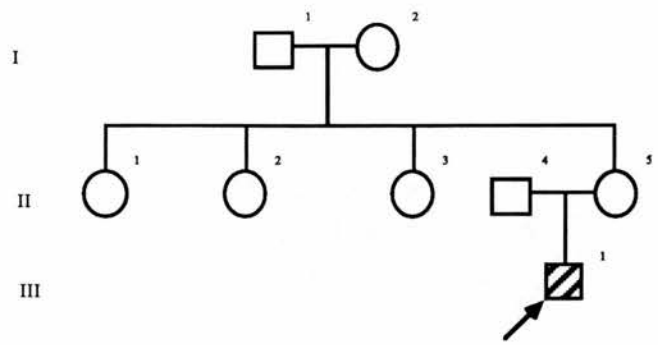
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-2	Obligate carrier	Carrier	Informative Bgl II: Obligate carrier	N/A	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the 2.8kb Bgl II allele. II-2 is an obligate carrier as she has 2 affected sons. Her coagulation phenotype is abnormal. She is informative for only the linked Bgl II polymorphism and although could be offered prenatal diagnosis there would be a 5% risk of error due to possible recombination.

Family No. 25

Pedigree.



Pedigree and Phenotype Data.

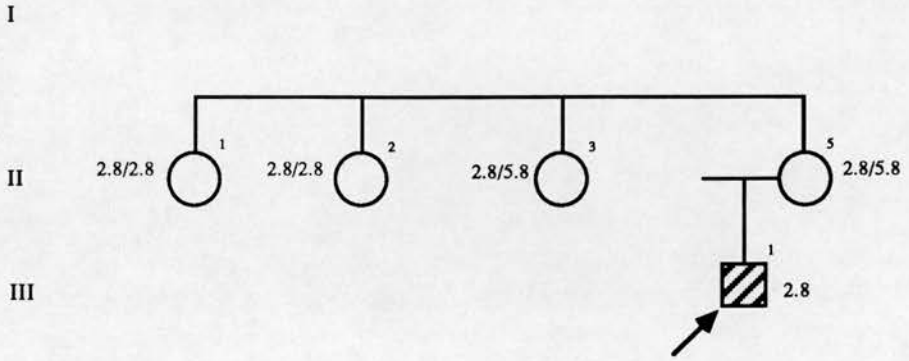
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-1	F	0.40	1.42	1.12	0.28	0.36	Potential carrier	Carrier
II-2	F	0.65	1.37	1.25	0.47	0.52	Potential carrier	Carrier
II-3	F	0.70	0.98	0.66	0.71	1.06	Potential carrier	Normal
II-5	F	0.75	1.05	1.25	0.71	0.60	Potential carrier	Normal
III-1	M	0.00	2.40	0.71			Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
II-1	F	0.8/0.8	5.0/5.0	4.8/4.8	2.8/2.8	Carrier or Normal
II-2	F	0.8/0.8	5.0/5.0	4.8/4.8	2.8/2.8	Carrier or Normal
II-3	F	0.8/0.8	-	4.8/4.8	2.8/5.8	Carrier or Normal
II-5	F	0.8/0.8	-	-	2.8/5.8	Potential carrier
III-1	M	0.8	-	-	2.8	Propositus

Family No. 25 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

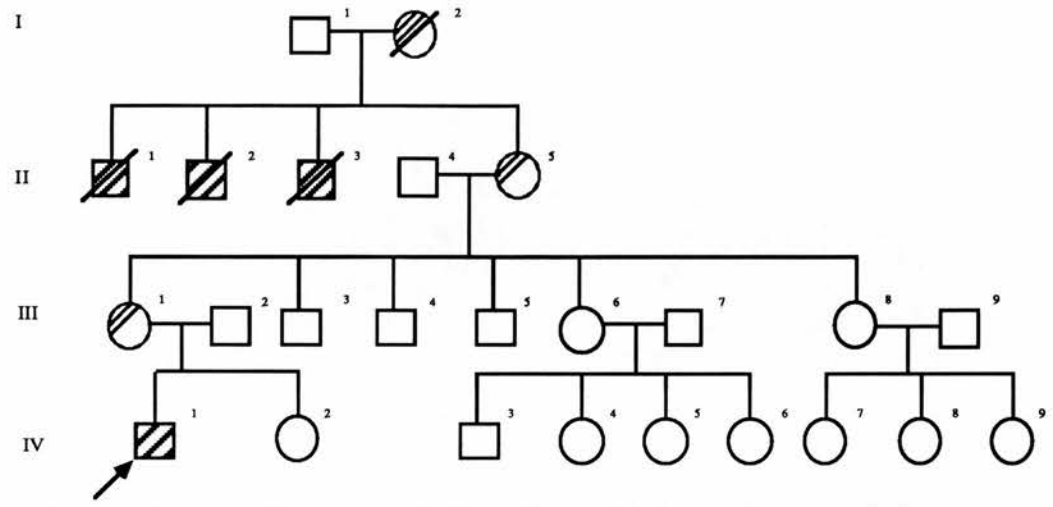
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-1	Potential carrier	Carrier	Heterozygous Bgl II: Potential carrier	Yes	N/A
II-2	Potential carrier	Carrier	Heterozygous Bgl II: Potential carrier	Yes	N/A
II-3	Potential carrier	Normal	Heterozygous Bgl II: Potential carrier	Yes	N/A
II-5	Potential carrier	Normal	Heterozygous Bgl II: Potential carrier	Yes	N/A

Implications and interpretations from these studies.

The mutant gene is marked by the 2.8kb allele of the Bgl II polymorphism which by implication must be derived from I-1 as this is the only common allele in his daughters. II-1, II-2, II-3 and II-5 are all potential carriers, although only II-1 and II-2 have abnormal coagulation phenotypes. The origin of the mutation in this family is unclear and although I-1 is clinically normal it is possible that he is a germ line mosaic and, therefore, all his daughters are at risk of being carriers. Differences in Lyonisation would explain the variations in VIII:C found in
II-1 - II-5. Ideally such a situation requires clarification by demonstrating the kindred specific mutation and screening of the 'at-risk' females for its presence or absence. II-5 could be offered prenatal diagnosis using the Bgl II polymorphism but would require confirmation of an affected pregnancy by fetal blood sampling.

Family No. 26

Pedigree.



Pedigree and Phenotype Data.

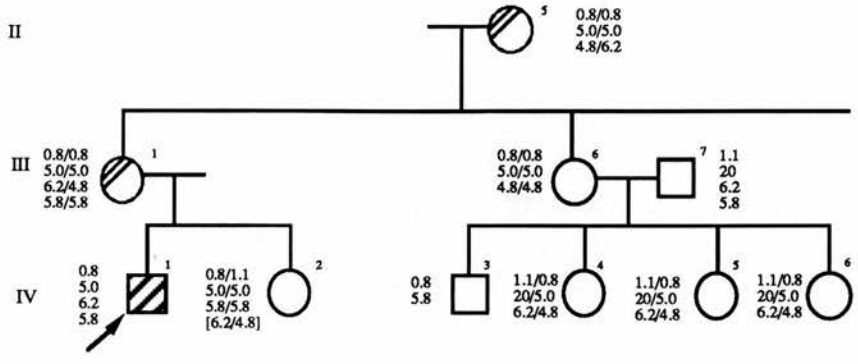
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-5	F	1.01	0.64	-	1.58	-	Obligate carrier	Normal
III-1	F	0.46	0.70	-	0.66	-	Obligate carrier	Carrier
III-6	F	1.83	1.42	-	1.29	-	Potential carrier	Normal
III-7	M	0.95	2.00	-	0.47	-		
IV-1	M	0.00	1.77	0.40	-	-	Propositus	
IV-2	F	1.20	0.64	0.35	1.87	3.40	Potential carrier	Normal
IV-3	M	0.64	2.10	-	0.30	-		
IV-4	F	0.69	0.70	-	0.99	-	Potential carrier	Normal
IV-5	F	0.56	0.45	-	1.24	-	Potential carrier	Normal
IV-6	F	0.98	0.88	-	1.11	-	Potential carrier	Normal

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
II-5	F	0.8/0.8	5.0/5.0	4.8/6.2	-	N/A - Obligate carrier
III-1	F	0.8/0.8	5.0/5.0	4.8/6.2	5.8/5.8	N/A - Obligate carrier
III-6	F	0.8/0.8	5.0/5.0	4.8/4.8	-	Normal
III-7	M	1.1	20.0	6.2	5.8	
IV-1	M	0.8	5.0	6.2	5.8	Propositus
IV-2	F	0.8/1.1	5.0/5.0	4.8/6.2	5.8/5.8	Potential carrier
IV-3	M	0.8	-	4.8/6.2	5.8	
IV-4	F	0.8/1.1	5.0/20.0	4.8/6.2	-	Normal
IV-5	F	0.8/1.1	5.0/20.0	4.8/6.2	-	Normal
IV-6	F	0.8/1.1	5.0/20.0	4.8/6.2	-	Normal

Family No. 26 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 10

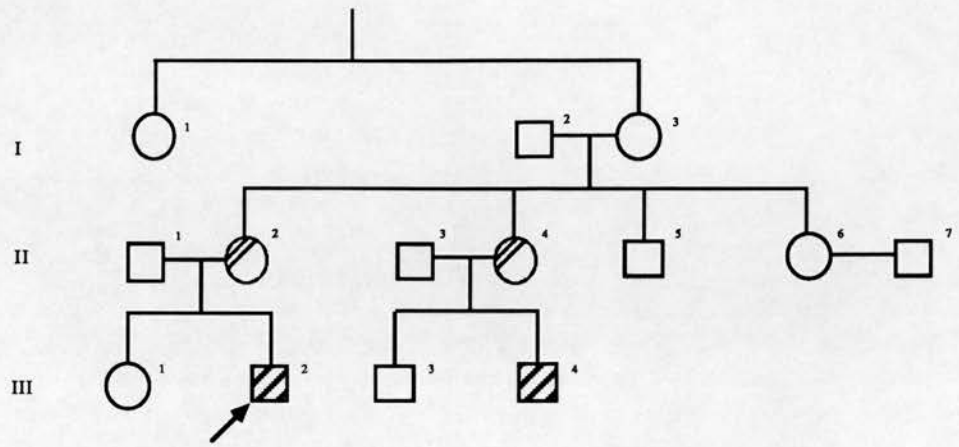
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-5	Obligate carrier	Normal	Informative Xba I: Obligate carrier	N/A	Yes
III-1	Obligate carrier	Carrier	Informative Xba I: Obligate carrier	N/A	Yes
III-6	Potential carrier	Normal	Normal	Yes	No
IV-2	Potential carrier	Normal	Heterozygous Bcl I/ Xba I: Potential carrier	Yes	Yes
IV-4	Potential carrier	Normal	Normal	Yes	N/A
IV-5	Potential carrier	Normal	Normal	Yes	N/A
IV-6	Potential carrier	Normal	Normal	Yes	N/A

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [0.8/5.0/6.2/5.8] haplotype. II-5 and III-1 are both obligate carriers although only III-1 has an abnormal phenotype. The haemophilic haplotype [0.8/5.0/6.2/5.8] has not been inherited by III-6 and therefore, excludes both herself and her offspring as carriers. The father of IV-2 was not available for study and her carrier status cannot, therefore, be established.

Family No. 27

Pedigree.



Pedigree and Phenotype Data.

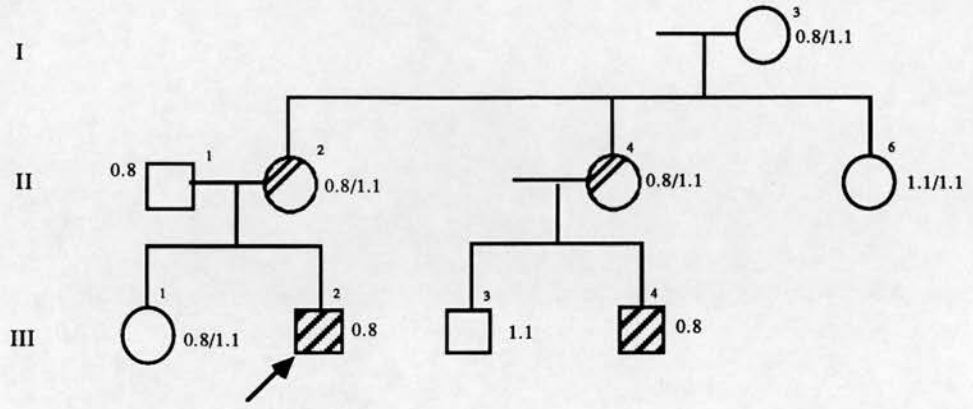
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-3	F	0.30	2.05	1.00	0.15	0.30	Potential carrier	Carrier
II-1	M	0.68	0.72	0.70	0.94	0.97		
II-2	F	1.00	1.90	1.74	0.53	0.57	Obligate carrier	Carrier
II-4	F	0.88	1.42	1.20	0.62	0.73	Obligate carrier	Normal
II-5	M	0.74	1.08	-	0.69	-		
II-6	F	1.05	2.10	1.25	0.50	0.84	Potential carrier	Carrier
III-1	F	0.94	1.10	1.25	0.85	0.75	Potential carrier	Normal
III-2	M	0.00	1.66	0.84	-	-	Propositus	
III-3	M	1.25	1.30	1.40	0.96	0.89		
III-4	M	0.00	1.05	1.18	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Bgl II	Carrier status from Genotype
I-3	F	0.8/1.1	5.0/20.0	5.8/5.8	Carrier
II-1	M	0.8	-	5.8	
II-2	F	0.8/1.1	-	5.8/5.8	N/A - Obligate carrier
II-4	F	0.8/1.1	-	5.8/5.8	N/A - Obligate carrier
II-6	F	1.1/1.1	5.0/20.0	5.8/5.8	Normal
III-1	F	0.8/1.1	-	5.8/5.8	Normal
III-2	M	0.8	-	5.8	Propositus
III-3	M	1.1	-	5.8	
III-4	M	0.8	-	5.8	Propositus

Family No. 27 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 9

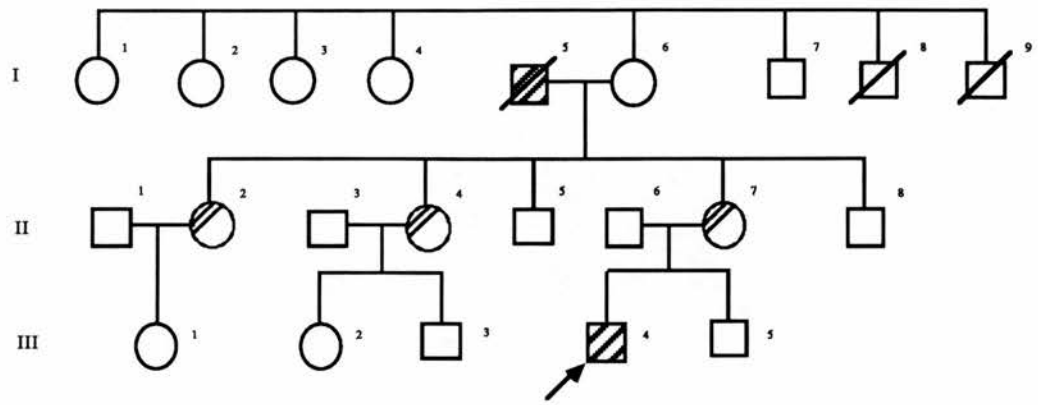
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-3	Potential carrier	Carrier	Informative Bcl I: Carrier	Yes	Yes
II-2	Obligate carrier	Carrier	Informative Bcl I: Obligate carrier	N/A	Yes
II-4	Obligate carrier	Normal	Informative Bcl I: Obligate carrier	N/A	Yes
II-6	Potential carrier	Normal	Normal	Yes	N/A
III-1	Potential carrier	Normal	Informative Bcl I: Normal	Yes	N/A

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the 0.8kb Bcl I allele. II-2 and II-4 are obligate carriers each having a single affected son and a family history of haemophilia. II-2 has a carrier phenotype but II-4 is normal. From the pedigree it is not clear whether the mutant gene has originated with the maternal grandfather or maternal grandmother although the coagulation data suggests the latter (I-3) and this is supported by genotypic analysis. From this data it is possible to confidently exclude II-6 as a carrier as she has not inherited the 0.8kb allele. In addition it is possible to exclude III-1 as a carrier as she has inherited the normal 1.1kb allele from her mother and the 0.8kb allele from her father. II-4 and II-2 are both informative for the Bcl I RFLP which could be used for prenatal diagnosis.

Family No. 28

Pedigree.



Pedigree and Phenotype Data.

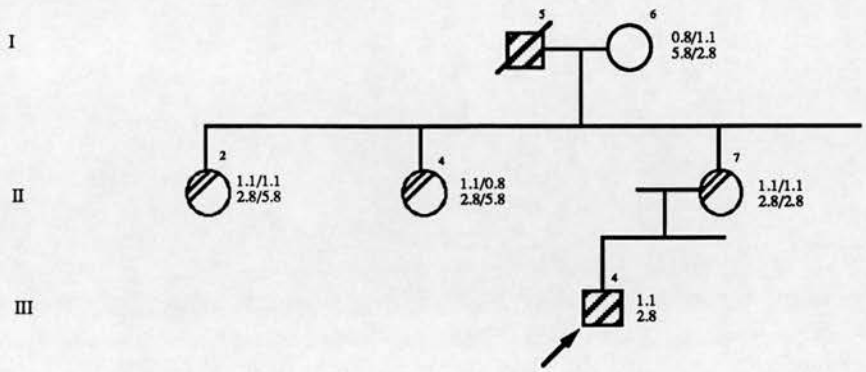
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-6	F	1.05	1.41	1.65	0.74	0.64	Normal	Normal
II-2	F	0.45	0.94	1.30	0.48	0.35	Obligate carrier	Carrier
II-4	F	0.32	1.30	1.20	0.25	0.27	Obligate carrier	Carrier
II-7	F	0.50	1.75	1.30	0.29	0.38	Obligate carrier	Carrier
III-4	M	0.05	-	-	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
I-6	F	0.8/1.1	-	-	2.8/5.8	N/A - Normal
II-2	F	1.1/1.1	20/20	6.2/6.2	2.8/5.8	N/A - Obligate carrier
II-4	F	0.8/1.1	-	-	2.8/5.8	N/A - Obligate carrier
II-7	F	1.1/1.1	20/20	6.2/6.2	2.8/2.8	N/A - Obligate carrier
III-4	M	1.1	20	6.2	2.8	Propositus

Family No. 28 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-6	Normal	N/A	N/A	N/A	N/A
II-2	Obligate carrier	Carrier	Informative Bcl I: Obligate carrier	N/A	Yes
II-4	Obligate carrier	Carrier	Informative Bcl I: Obligate carrier	N/A	Yes
II-7	Obligate carrier	Carrier	Informative Bcl I: Obligate carrier	N/A	Yes

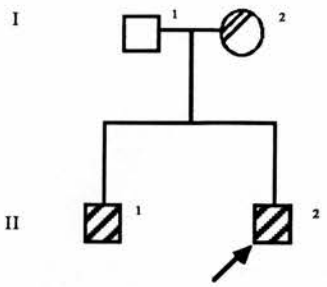
Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [1.1/2.8] Bcl I/Bgl II haplotype. I-6 is a normal female. II-2, II-4 and II-7 are all obligate carriers being the daughters of a severely affected haemophiliac and all have a coagulation phenotype consistent with carriership. They are all informative for the Bcl I polymorphism and can, therefore, offered prenatal diagnosis.

Analysis of the family using the Bcl I and Bgl II RFLP's shows a recombination between I-6 and II-2. The [1.1/2.8] haplotype is common to both II-2, II-4 and II-7 and is derived from I-5. II-4 and II-7 have inherited either a [0.8/5.8] or a [1.1/2.8] haplotype from their mother but II-2 has inherited a [1.1/5.8] indicating a cross-over between the Bcl I and Bgl II loci. Non-paternity to explain these findings has not been excluded.

Family No. 29

Pedigree.



Pedigree and Phenotype Data.

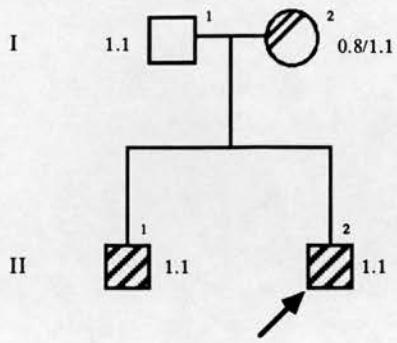
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-1	M	1.00	1.00	1.00	1.00	1.00		
I-2	F	0.60	1.00	1.40	0.60	0.43	Obligate carrier	Normal
II-1	M	0.00	1.10	0.67	-	-	Propositus	
II-2	M	0.00	1.60	1.50	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl II	Carrier status from Genotype
I-1	M	1.1	5.8	
I-2	F	0.8/1.1	5.8/5.8	N/A - Obligate carrier
II-1	M	1.1	5.8	Propositus
II-2	M	1.1	5.8	Propositus

Family No. 29 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

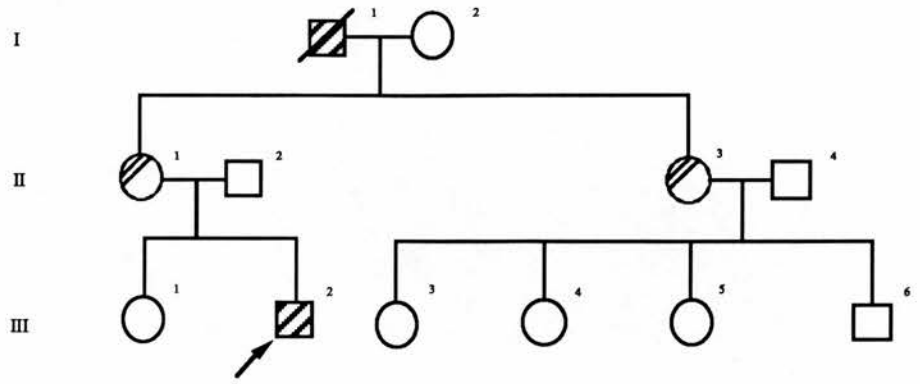
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Obligate carrier	Normal	Informative Bcl I: Obligate carrier	N/A	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the 1.1kb allele of the Bcl I RFLP. I-2 is an obligate carrier having 2 affected sons although a normal coagulation phenotype. Unfortunately, she is adopted and no previous family history was obtainable. However, she is informative for the Bcl I polymorphism and can be offered prenatal diagnosis if requested.

Family No. 30

Pedigree.



Pedigree and Phenotype Data.

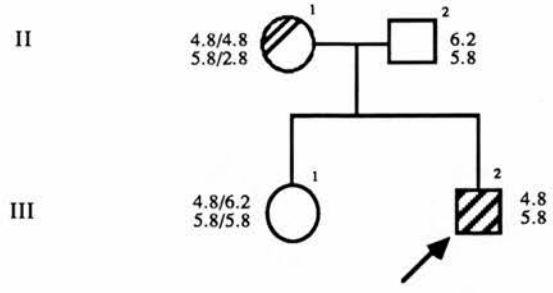
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-1	F	0.41	0.76	2.20	0.54	0.19	Obligate carrier	Carrier
II-2	M	1.00	-	-	-	-		
III-1	F	0.41	1.10	0.46	0.37	0.89	Potential carrier	Carrier
III-2	M	0.01	-	-	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
II-1	F	0.8/0.8	5.0/5.0	4.8/4.8	2.8/5.8	N/A - Obligate carrier
II-2	M	0.8	5.0	6.2	5.8	
III-1	F	0.8/0.8	5.0/5.0	4.8/6.2	5.8/5.8	Carrier
III-2	M	0.8	5.0	4.8	5.8	Propositus

Family No. 30 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

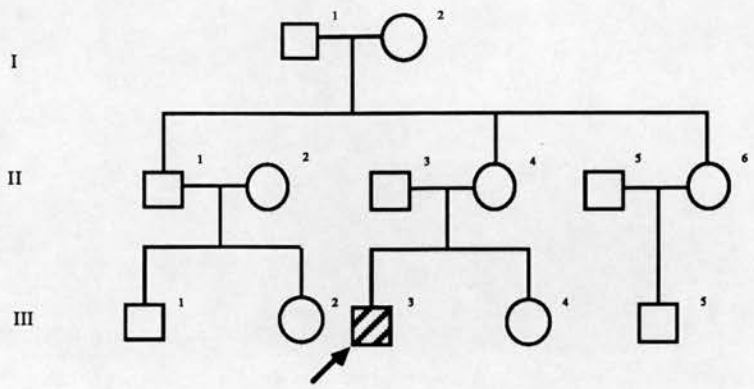
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-1	Obligate carrier	Carrier	Informative Bgl II: Obligate carrier	N/A	Yes
III-1	Potential carrier	Carrier	Informative Xba I: Carrier	Yes	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [4.8/5.8] Xba I/Bgl II haplotype. II-1 is an obligate carrier and has a carrier phenotype. III-1 is a potential carrier and has a carrier phenotype. III-1 has inherited the haemophiliac haplotype [4.8/5.8] from her mother and is therefore, a carrier. II-1 is informative for the Bgl II polymorphism and III-1 for the Xba I polymorphism and both, therefore, could be offered prenatal diagnosis if requested.

Family No. 31

Pedigree.



Pedigree and Phenotype Data.

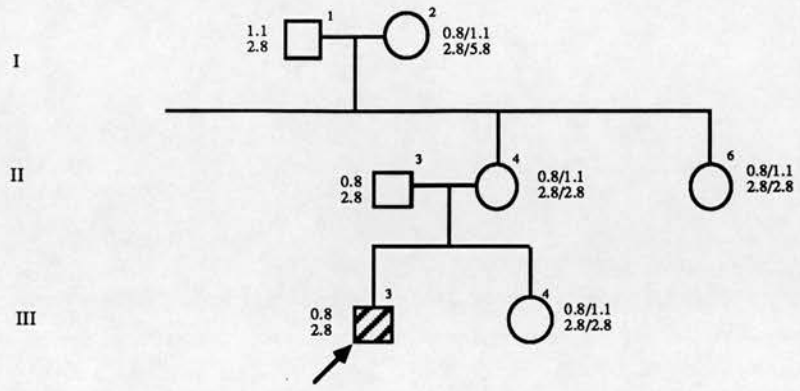
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-1	M	0.72	0.50	0.80	1.40	0.90		
I-2	F	0.30	0.66	0.69	0.45	0.43	Potential carrier	Carrier
II-4	F	0.82	0.69	0.65	1.19	1.26	Potential carrier	Normal
II-6	F	0.56	0.76	1.40	0.74	0.40	Potential carrier	Normal
III-3	M	0.00	0.74	0.62	-	-		
III-4	F	1.00	0.70	0.70	1.43	1.43	Potential carrier	Normal

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Bgl II	Carrier status from Genotype
I-1	M	1.1	-	2.8	
I-2	F	0.8/1.1	5.0/5.0	2.8/5.8	Carrier
II-3	M	0.8	-	2.8	
II-4	F	0.8/1.1	-	2.8/2.8	Potential carrier
II-6	F	0.8/1.1	-	2.8/2.8	Carrier
III-3	M	0.8	5.0	2.8	
III-4	F	0.8/1.1	-	2.8/2.8	Normal

Family No. 31 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 7

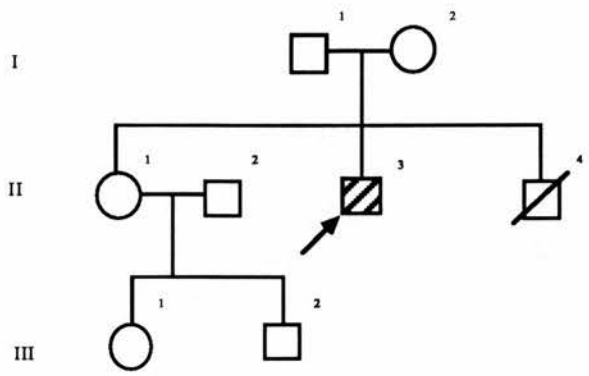
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Potential carrier	Carrier	Heterozygous Bcl I/ Bgl II: Carrier	N/A	Yes
II-4	Potential carrier	Normal	Heterozygous Bcl I: Carrier	N/A	Yes
II-6	Potential carrier	Normal	Heterozygous Bcl I: Carrier	Yes	Yes
III-4	Potential carrier	Normal	Heterozygous Bcl I: Normal	Yes	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [0.8/2.8] Bcl I/Bgl II haplotype and genotypic analysis shows that this is derived from I-2. I-2 is a potential carrier and has an abnormal coagulation phenotype.
Assuming I-2 to be a carrier then II-6 has inherited the haemophilic haplotype and is, therefore a carrier. III-4 is also a potential carrier although her coagulation phenotype is normal. She has inherited the non-haemophilic haplotype [1.1/2.8] from her mother (II-4) and is therefore normal. I-2, II-4 and II-6 are all informative for the Bcl I polymorphism and can be offered prenatal diagnosis if requested.

Family No. 32

Pedigree.



Pedigree and Phenotype Data.

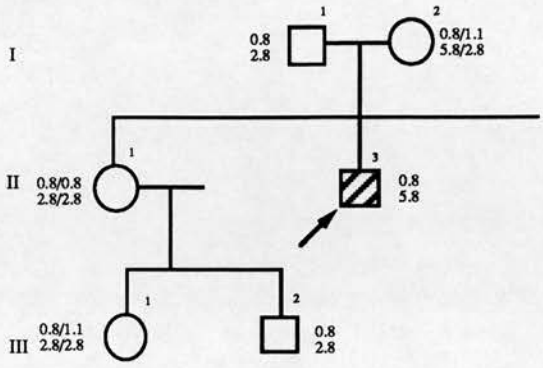
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-2	F	0.50	1.02	-	0.49	-	Potential carrier	Carrier
II-1	F	0.60	0.64	0.68	0.94	0.88	Potential carrier	Normal
II-3	M	0.00	-	-	-	-	Propositus	
III-1	F	0.70	0.91	0.80	0.77	0.87	Potential Carrier	Normal
III-2	M	No data available but clinically normal						

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Bgl II	Carrier status from Genotype
I-2	F	0.8/1.1	5.0/5.0	2.8/5.8	Potential carrier
II-1	F	0.8/0.8	5.0/5.0	2.8/2.8	Potential carrier
II-3	M	0.8	5.0	5.8	Propositus
III-1	F	0.8/1.1	5.0/5.0	2.8/2.8	Potential carrier
III-2	M	0.8	5.0	2.8	

Family No. 32 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 6

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Potential carrier	Carrier	Heterozygous Bcl I/ Bgl II: Potential carrier	No	Yes
II-1	Potential carrier	Normal	Non-informative: Potential carrier	Yes	No
III-1	Potential carrier	Normal	Heterozygous Bcl I/ Potential carrier	Yes	Yes

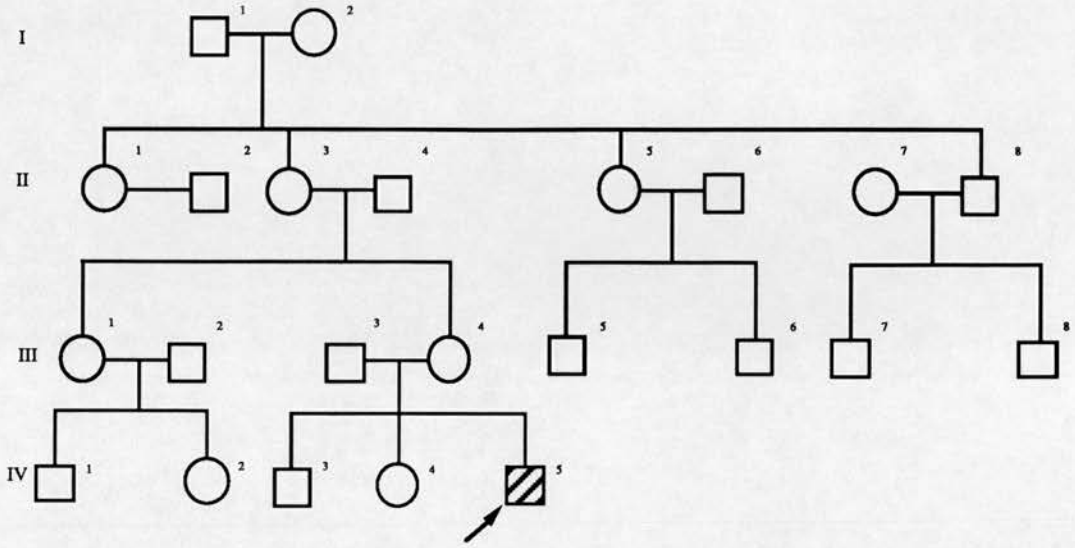
Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [0.8/5.8] Bcl I/Bgl II haplotype. I-2 is a potential carrier and has an abnormal coagulation phenotype. II-1 is a potential carrier although her coagulation phenotype is normal.
Establishing the carrier status of II-1 using the linked Bgl II polymorphisms suggests she is normal as she has inherited the 'normal' 2.8kb Bgl II allele. However, the intragenic Bcl I polymorphism indicates she is a carrier as she has inherited the 'abnormal' 0.8kb allele. This difference represents a cross-over or recombination between the Bcl I and Bgl II loci and again emphasises the problems associated with the use of linked RFLP's. Non-paternity to explain these findings has not been excluded.

The carrier status of III-1 cannot be established from the available data and ideally an informative polymorphism in her mother (II-1) should be sought.

Family No. 33

Pedigree.



Pedigree and Phenotype Data.

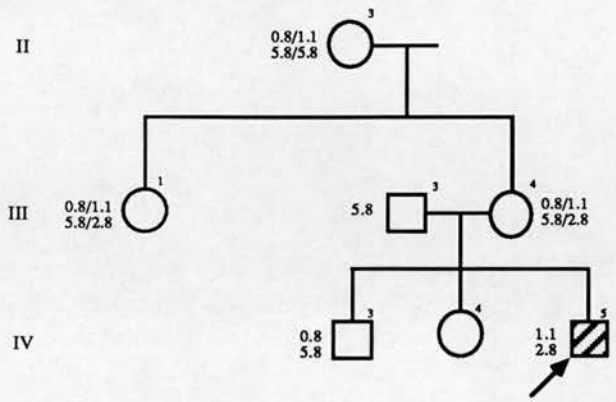
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-3	F	1.00	1.65	1.30	0.61	0.77	Potential carrier	Normal
III-1	F	1.00	1.45	1.50	0.69	0.67	Potential carrier	Normal
III-4	F	1.10	1.25	1.30	0.88	0.85	Potential carrier	Normal
III-3	M	1.20	0.78	0.86	1.54	1.40		
IV-3	M	1.00	0.90	0.94	1.11	1.06		
IV-4	F	1.00	0.86	0.86	1.16	1.16	Potential carrier	Normal
IV-5	M	0.00	0.82	-	0.00	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl II	Carrier status from Genotype
II-3	F	0.8/1.1	5.8/5.8	Normal
III-1	F	0.8/1.1	2.8/5.8	Normal
III-4	F	0.8/1.1	2.8/5.8	Potential carrier
III-3	M	-	5.8	
IV-3	M	0.8	5.8	
IV-4	F	No data available		
IV-5	M	1.1	2.8	Propositus

Family No. 33 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 6

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-3	Potential carrier	Normal	Heterozygous Bcl I: Potential carrier	Unhelpful	Yes
III-1	Potential carrier	Normal	Heterozygous Bcl I/ Bgl II: Potential carrier	Unhelpful	Yes
III-4	Potential carrier	Normal	Heterozygous Bcl I: Potential carrier	N/A	Yes

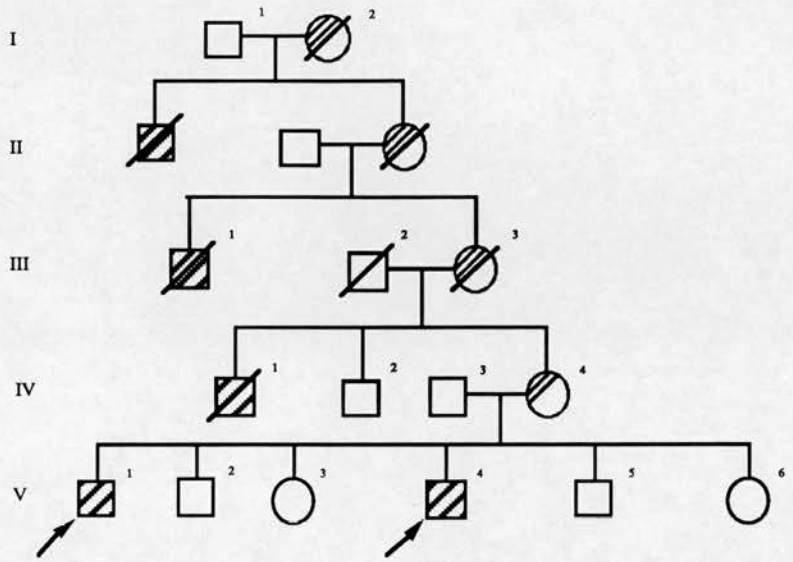
Implications and interpretations from these studies.

The haemophilic haplotype [1.1/2.8] has been inherited from the maternal grandfather. Although clinically normal it is possible that he is a germ line mosaic in which case there is a risk that III-1 is a carrier.

Although the coagulation phenotype is normal in III-1 and III-4, ideally the mutation in the FVIII gene of IV-5 should be characterised and used to clarify carrier status in the 'at-risk' females.

Family No. 34

Pedigree.



Pedigree and Phenotype Data.

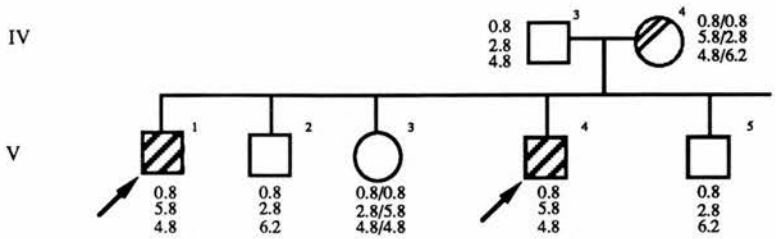
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
IV-3	M	1.05	1.28	1.40	0.82	0.75		
IV-4	F	0.40	0.41	0.22	0.98	0.18	Obligate carrier	Carrier
V-1	M	0.00	-	-	-	-	Propositus	
V-2	M	0.80	0.74	-	1.08	-		
V-3	F	0.70	0.56	0.55	1.25	1.27	Potential carrier	Normal
V-4	M	0.00	-	-	-	-	Propositus	
V-5	M	No data	-	-	-	-	Clinically normal	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl II	Xba I	Carrier status from Genotype
IV-3	M	0.8	2.8	4.8	
IV-4	F	0.8/0.8	5.8/2.8	4.8/6.2	N/A - Obligate carrier
V-1	M	0.8	5.8	4.8	Propositus
V-2	M	0.8		6.2	
V-3	F	0.8/0.8	2.8/5.8	4.8/4.8	Carrier
V-4	M	0.8	5.8	4.8	Propositus
V-5	M	0.8	2.8	6.2	

Family No. 34 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 7

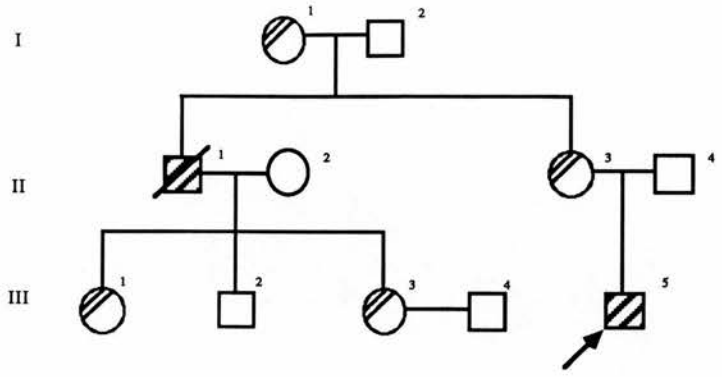
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
IV-4	Obligate carrier	Carrier	Informative Xba I/ Bgl II: Obligate carrier	N/A	Yes
V-3	Potential carrier	Normal	Informative Bgl II: Carrier	Yes	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [0.8/2.8/6.2] haplotype. IV-4 is an obligate carrier and has an abnormal coagulation phenotype. She is informative for both the Bgl II and Xba I polymorphisms and can be offered prenatal diagnosis if requested. V-3 has inherited the haemophilic haplotype [4.8/5.8] from her mother and is, therefore, a carrier. She is heterozygous for the Bgl II RFLP which could be used for prenatal diagnosis.

Family No. 35

Pedigree.



Pedigree and Phenotype Data.

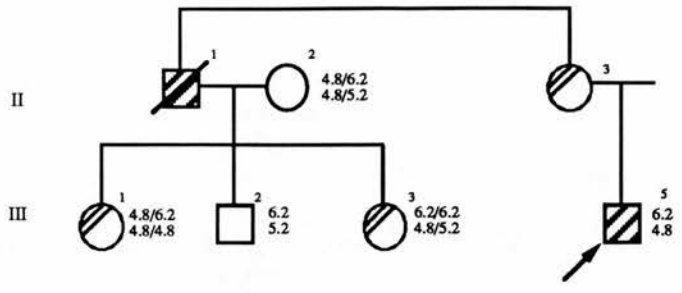
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-2	F	-	-	-	-	-	Normal	N/A
III-1	F	0.35	0.64	-	0.55	-	Obligate carrier	Carrier
III-2	M	1.20	-	-	-	-		
III-3	F	0.73	0.64	-	1.14	-	Obligate carrier	Normal
III-5	M	0.01	-	-	-	-	Propositus	

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Taq I	Carrier status from Genotype
II-2	F	0.8/0.8	5.0/5.0	4.8/6.2	5.8/5.8	4.8/5.2	N/A
III-1	F	0.8/0.8	5.0/5.0	4.8/6.2	5.8/5.8	4.8/4.8	N/A - Obligate carrier
III-2	M	0.8	5.0	6.2	5.8	5.2	
III-3	F	0.8/0.8	5.0/5.0	6.2/6.2	5.8/5.8	4.8/5.2	N/A - Obligate carrier
III-5	M	0.8	5.0	6.2	5.8	4.8	Propositus

Family No. 35 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

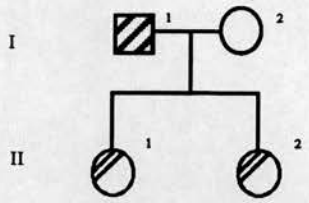
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-2	Normal	N/A	N/A	N/A	N/A
III-1	Obligate carrier	Carrier	Informative Xba I: Obligate carrier	N/A	Yes
III-3	Obligate carrier	Normal	Informative Taq I: Obligate carrier	N/A	Yes

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [4.8/6.2] Xba I/Taq I haplotype.
III-1 and III-3 are obligate carriers as both are the daughters of a severely affected haemophiliac (now deceased) although only III-1 has a carrier phenotype. III-1 is informative for the intragenic Xba I polymorphism whilst III-3 is homozygous for all markers except the linked Taq I polymorphism. Both could be offered prenatal diagnosis but in the case of III-3 there would be a 15% chances of error due to three possible recombinations.

Family No. 36

Pedigree.



Pedigree and Phenotype Data.

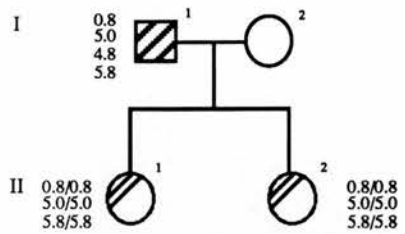
Gen. No	Sex		Carrier status from: Pedigree
I-1	M	No coagulation data	Propositus
I-2	F	No coagulation data	Normal
II-1	F	No coagulation data	Obligate carrier
II-2	F	No coagulation data	Obligate carrier

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
I-1	M	0.8	5.0	4.8	5.8/5.8	Propositus
I-2	F	No Data available				
II-1	F	0.8/0.8	5.0/5.0		5.8/5.8	N/A - Obligate carrier
II-2	F	0.8/0.8	5.0/5.0		5.8/5.8	N/A - Obligate carrier

Family No. 36 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 3

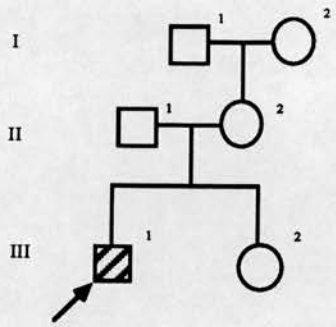
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
III-1	Obligate carrier	No data	Non-informative: Obligate carrier	N/A	No
III-2	Obligate carrier	No data	Non-informative: Obligate carrier	N/A	No

Implications and interpretations from these studies.

II-1 and II-2 are both obligate carriers being the daughters of an affected haemophiliac. They were non-informative for the three polymorphisms analysed and insufficient DNA was available to study the remainder.

Family No. 37

Pedigree.



Pedigree and Phenotype Data.

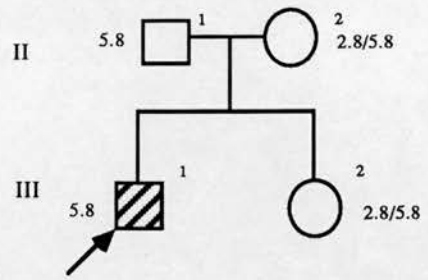
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-1	M	No data available - clinically normal						
II-2	F	0.65	1.02	0.56	0.64	1.16	Potential carrier	Normal
III-1	M	0.09	0.92	1.16	-	-	Propositus	
III-2	F	1.80	1.60	1.10	1.12	1.64	Potential carrier	Normal

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
II-1	M	0.8		4.8	5.8	
II-2	F	0.8/0.8	5.0/5.0	4.8/4.8	2.8/5.8	Potential carrier
III-1	M	0.8	5.0	4.8	5.8	Propositus
III-2	F	0.8/0.8		4.8	2.8/5.8	Normal

Family No. 37 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-2	Potential carrier	Normal	Heterozygous Bgl II: Potential carrier	N/A	Yes
III-2	Potential carrier	Normal	Heterozygous Bgl II: Normal	Yes	Yes

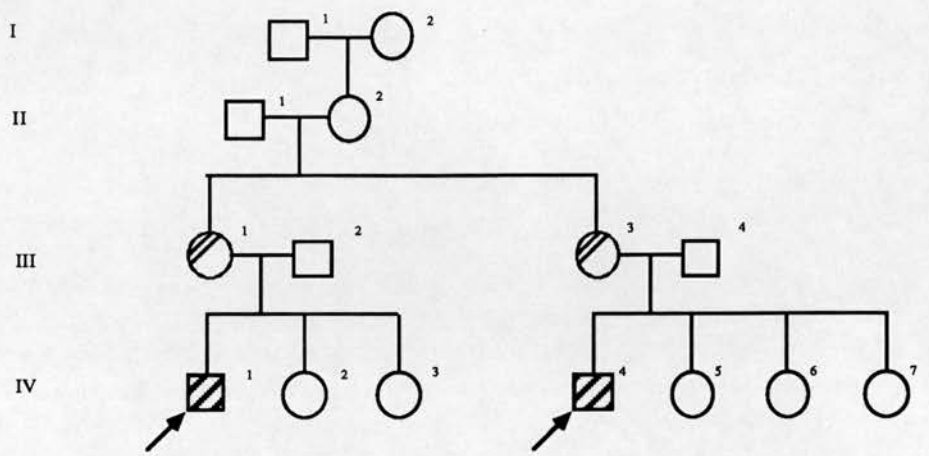
Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the 5.8kb allele of the Bgl II RFLP.
II-2 and III-2 are potential carriers and both have a normal coagulation phenotype. III-2 has inherited a normal 2.8kb allele from her mother and a normal 5.8kb allele from her father and is, therefore, not a carrier. There is a 10% chance of error due to two possible recombinations.

This family illustrates that in sporadic haemophilia, haplotype exclusion can be extremely important in determining the carrier status of 'at-risk' women.

Family No. 38

Pedigree.



Pedigree and Phenotype Data.

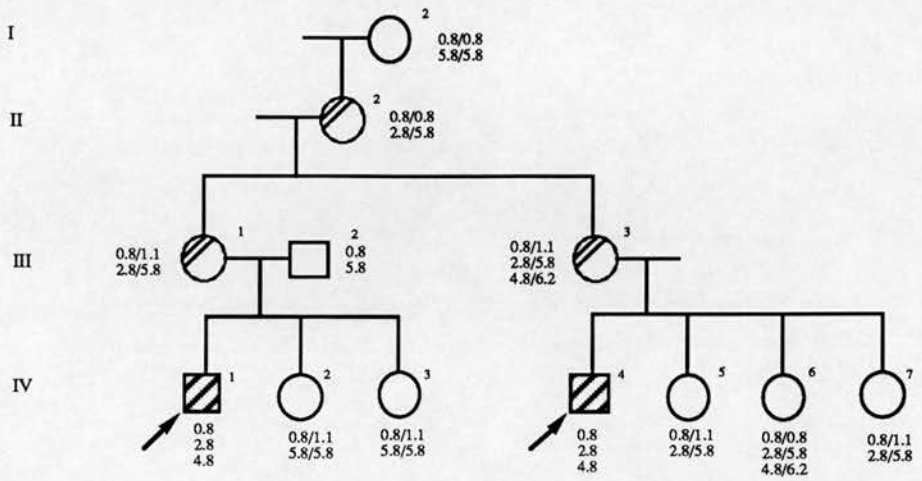
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-2	F	No data available-		-	-	-	Potential carrier	
II-2	F	No data available		-	-	-	Potential carrier	
III-1	F	0.58	0.66	1.12	0.88	0.52	Obligate carrier	Normal
III-2	M	1.60	1.15	0.97	1.39	1.65		
III-3	F	1.00	1.00	1.40	1.00	0.71	Obligate carrier	Normal
IV-1	M	0.01	1.20	-	-	-	Propositus	
IV-2	F	1.50	0.90	0.92	1.67	1.63	Potential carrier	Normal
IV-3	F	1.50	0.84	0.90	1.79	1.67	Potential carrier	Normal
IV-4	M	0.01	-	-	-	-	Propositus	
IV-5	F	1.70	1.10	1.40	0.55	1.21	Potential carrier	Carrier
IV-7	F	2.00	1.80	0.78	1.11	2.56	Potential carrier	Normal

Genotype Data.

Gen. No	Sex	Bcl I	Xba I	Bgl II	Carrier status from Genotype
I-2	F	0.8/0.8	-	5.8/5.8	Potential carrier
II-2	F	0.8/0.8	-	2.8/5.8	Potential carrier
III-1	F	0.8/1.1	-	2.8/5.8	N/A - Obligate carrier
III-2	M	0.8	-	5.8	
III-3	F	0.8/1.1	4.8/6.2	2.8/5.8	N/A - Obligate carrier
IV-1	M	0.8	4.8	2.8	Propositus
IV-2	F	0.8/1.1	-	5.8/5.8	Normal
IV-3	F	0.8/1.1	-	5.8/5.8	Normal
IV-4	M	0.8	-	2.8	Propositus
IV-5	F	0.8/1.1	-	2.8/5.8	Normal
IV-7	F	0.8/1.1	-	2.8/5.8	Normal

Family No. 38 (continued)

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 11

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic data	Applications of genotypic data to: CD	AND
I-2	Potential carrier	No data	Non-informative: Potential carrier	No	Yes
II-2	Potential carrier	No data	Heterozygous Bgl II: Potential carrier	No	Yes
III-1	Carrier	Normal	Informative Bcl I/ Xba I/Bgl II: Obligate carrier	N/A	Yes
III-3	Carrier	Normal	Informative Bcl I/ Xba I/Bgl II: Obligate carrier	N/A	Yes
IV-2	Potential carrier	Normal	Heterozygous Bcl I: Normal	Yes	N/A
IV-3	Potential carrier	Normal	Heterozygous Bcl I: Normal	Yes	N/A
IV-5	Potential carrier	Normal	Heterozygous Bcl I: Normal	Yes	N/A
IV-7	Potential carrier	Normal	Heterozygous Bcl I: Normal	Yes	N/A

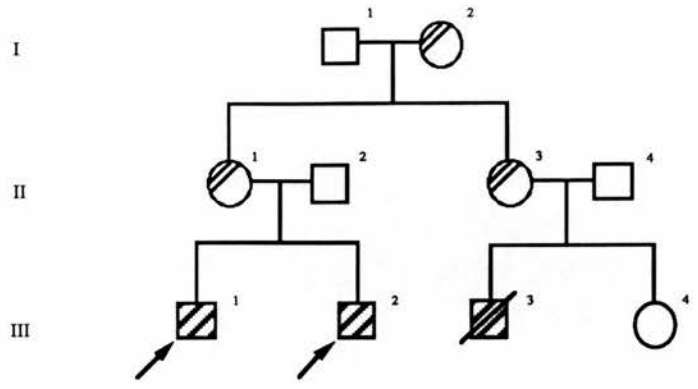
Pedigree No. 38 (continued).

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is marked by the [0.8/2.8/4.8] Bcl I/Bgl II/Xba I haplotype. III-1 and III-3 are both obligate carriers although their coagulation phenotypes are normal. The haemophilic haplotype has not been inherited by IV-2, IV-3, IV-5 or IV-7 and they are, therefore, normal. Both III-1 and III-3 are informative for the Bcl I and Xba I polymorphisms and can be offered prenatal diagnosis if requested.

Family No. 39

Pedigree.



Pedigree and Phenotype Data.

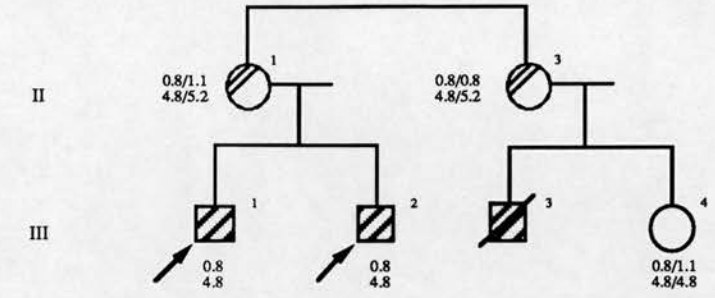
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-1	F	0.72	0.68	0.86	1.06	0.84	Obligate carrier	Normal
II-3	F	0.15	0.74	0.86	0.20	0.17	Obligate carrier	Carrier
III-1	M	0.00	1.24	1.10	-	-	Propositus	
III-2	M	0.00	1.36	1.34	-	-	Propositus	
III-4	F	0.92	0.86	0.76	1.07	1.21	Potential carrier	Normal

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Taq I	Carrier status from Genotype
II-1	F	0.8/0.8	-	-	5.8/5.8	4.8/5.25	N/A - Obligate carrier
II-3	F	0.8/0.8	5.0/5.0	4.8/4.8	5.8/5.8	4.8/5.2	N/A - Obligate carrier
III-1	M	0.8	5.0	4.8	5.8	4.8	Propositus
III-2	M	0.8	5.0	4.8	5.8	4.8	Propositus
III-4	F	0.8/1.1	-	-	5.8/5.8		Potential carrier

Family No. 39 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

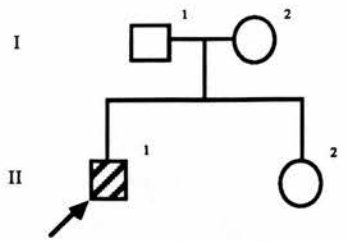
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-1	Obligate carrier	Normal	Informative Bcl I: Obligate carrier	N/A	Yes
II-3	Obligate carrier	Carrier	Informative Taq I: Obligate carrier	N/A	Yes
III-4	Potential carrier	Normal	Non-informative: Potential carrier	No	Yes

Implications and interpretations from these studies.

In this family the haemophilic gene is marked by the [0.8/4.8] Bcl I/Taq I haplotype.
II-1 and II-3 are both obligate carriers although only II-3 has an abnormal phenotype. II-1 is informative for the Bcl I polymorphism and I-3 for the Taq I polymorphism. No data was available on the father of III-4.
This family illustrates the differences in Lyonisation which can occur in obligatory carriers (II-1 VIII:C 0.72; II-3 VIII:C 0.15) and highlights the problems in phenotypic based methods of carrier detection.
The finding of identical Taq I alleles in II-1 and II-3 but different Bcl I alleles suggests either I-2 is homozygous [5.2/5.2] or a recombination has occurred between the Bcl I and Taq I loci. DNA was not available on the maternal grandparents to clarify this.
An alternative explanation and more plausible for these findings is non-paternity. As II-I and II-3 are both obligate carriers and their putative father is normal, the difference in their Bcl I markers suggests they have different fathers as the maternal Bcl I marker (she must be an obligate carriers) should be the same in the two women. These findings emphasise the necessity for paternity testing in RFLP studies.

Family No. 40

Pedigree.



Pedigree and Phenotype Data.

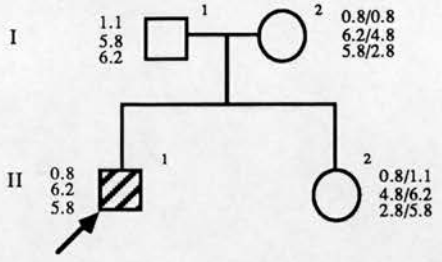
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
I-1	M	0.83	0.90	1.00	0.92	0.83		
I-2	F	0.43	0.66	0.79	0.65	0.54	Potential carrier	Carrier
II-1	M	0.00	1.00	-	-	-	Propositus	
II-2	F	1.00	0.90	0.77	1.11	1.30	Potential carrier	Normal

Genotype Data.

Gen. No	Sex	Bcl I	Bgl I	Xba I	Bgl II	Carrier status from Genotype
I-1	M	1.1	5.0	6.2	5.8	Propositus
I-2	F	0.8/0.8	5.0	4.8/6.2	2.8/5.8	Potential carrier
II-1	M	0.8	5.0	6.2	5.8	
II-2	F	0.8/1.1	5.0/5.0	4.8/6.2	2.8/5.8	Normal

Family No. 40 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

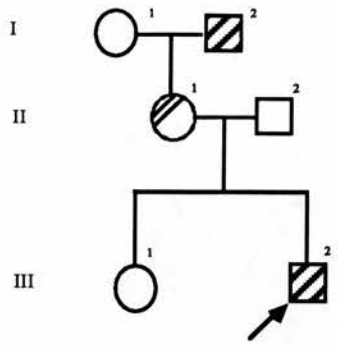
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Potential carrier	Carrier	Heterozygous Xba I/ Bgl II: Potential carrier	No	Yes
II-2	Potential carrier	Normal	Heterozygous Xba I/ Bgl II/ Bcl I: Normal	Yes	N/A

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is associated with the [0.8/6.2/5.8] Bcl I/Xba I/Bgl II haplotype. I-2 and II-2 are both potential carriers although only I-2 has an abnormal coagulation phenotype. Genotypic analysis shows the haemophilic haplotype [0.5/6.2/5.8] has not been inherited by II-2 who is, therefore, normal. Prenatal diagnosis can be offered to I-2 using the Xba I polymorphism.

Family No. 41

Pedigree.



Pedigree and Phenotype Data.

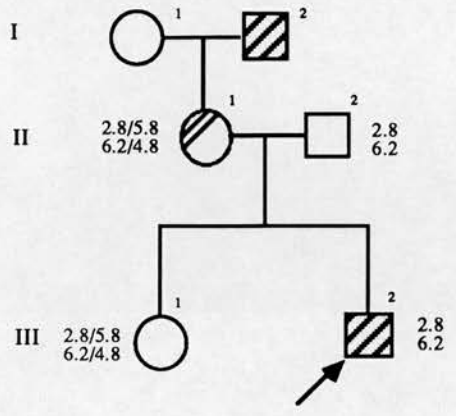
Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF:RCo	Carrier status from: Pedigree	Phenotype
II-2	M	-	-	-	-	-		
II-1	F	0.34	1.50	-	0.23	-	Obligate carrier	Carrier
III-2	M	0.00	-	-	-	-	Propositus	
III-1	F	1.30	1.00	-	1.30	-	Potential carrier	Normal

Genotype Data.

Gen. No	Sex	Bcl I	Xba I	Bgl II	Carrier status from Genotype
II-2	M	0.8	6.2	2.8	
II-1	F	0.8/0.8	4.8/6.2	2.8/5.8	N/A - Obligate carrier
III-2	M	0.8	6.2	2.8	Propositus
III-1	F	0.8/0.8	4.8/6.2	2.8/5.8	Normal

Family No. 41 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

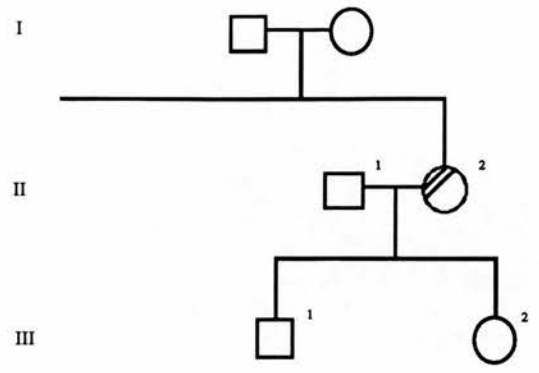
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-1	Obligate carrier	Carrier	Informative Xba I/ Bgl II: Obligate carrier	N/A	Yes
III-1	Potential carrier	Normal	Heterozygous Xba I/ Bgl II: Normal	Yes	N/A

Implications and interpretations from these studies.

In this pedigree the haemophilic gene is associated with the [2.8/6.2] Bgl II/Xba I haplotype. II-1 is an obligate carrier as she is the daughter of a haemophiliac. III-1 is a potential carrier but has a normal coagulation phenotype. She has inherited the normal [5.8/4.8] haplotype from her mother rather that the haemophilic [2.8/6.2] haplotype and is therefore normal. II-1 is informative for the Xba I polymorphism and can be offered prenatal diagnosis if requested.

Family No. 42

Pedigree.



Pedigree and Phenotype Data.

Gen. No	Sex	VIII:C	vWF:Ag	vWF: RCo	VIII:C/ vWF:Ag	VIII:C/ vWF	Carrier status from: Pedigree	Phenotype
II-2	F	0.18	0.80	-	0.23	-	*Carrier	Carrier
III-1	M	0.87	0.85	-	1.0	-		
III-2	F	0.38	0.57	-	0.67	-	Potential carrier	Carrier

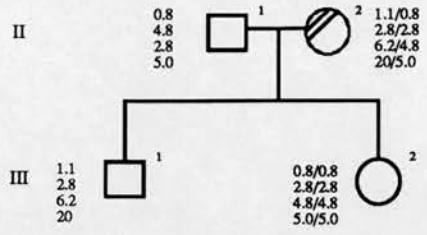
Genotype Data.

Gen.	Sex No	Bcl I	Xba I	Bgl I	Bgl II	Carrier status from Genotype
II-1	M	0.8	4.8	5.0	2.8	
II-2	F	0.8/1.1	4.8/6.2	5.0/20	2.8/2.8	Propositus
III-1	M	1.1	6.2	20	2.8	
III-2	F	0.8/0.8	4.8/4.8	5.0/5.0	2.8/2.8	Carrier

*** See following page and text.**

Family No. 42 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-2	-	?Carrier	Unhelpful	N/A	?
III-2	Potential Carrier	?Carrier	?Carrier	N/A	?

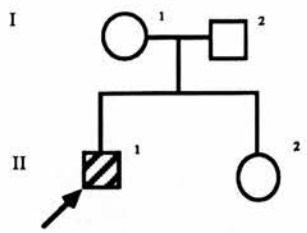
Implications and interpretations from these studies.

II-2 was found to have an abnormal coagulation profile consistent with carriership of haemophilia A, although there was no positive family history. The possibility of an unusual form of von Willebrand's disease affecting the VIII:C binding site has not been excluded. Studies to clarify this is are currently in progress.

From RFLP analysis, as II-1 is normal the other maternal haplotype [0.8/2.8/4.8] must be associated with the proposed haemophilic gene and as III-2 has inherited this we can infer that if II-2 is a carrier of haemophilia A then so is her daughter - a finding supported by the abnormal coagulation phenotype. These findings do not exclude vWD - if vWF binding studies are normal then identification of any mutations in the FVIII gene should be attempted. Although the genotypic and phenotypic data in this family are in agreement, variant von Willebrand's disease must be excluded before accurate genetic counselling can be given.

Family No. 43

Pedigree.



Pedigree and Phenotype Data.

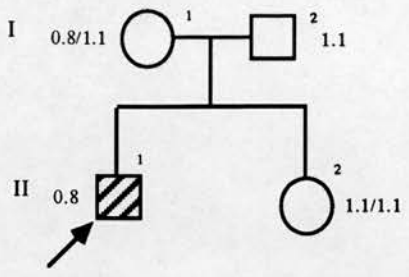
Gen. No	Sex		Carrier status from: Pedigree
I-1	F	No data available	Potential carrier
I-2	M	No data available	Normal
II-1	M	0.01	Propositus
II-2	F	No data available	Potential carrier

Genotype Data.

Gen. No	Sex	Bcl I	Carrier status from Genotype
I-1	F	0.8/1.1	Potential carrier
I-2	M	1.1	
II-1	M	0.8	Propositus
II-2	F	1.1/1.1	Normal

Family No. 43 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-1	Potential carrier	No Data	Heterozygous Bcl I: Potential carrier	N/A	Yes
II-2	Potential carrier-	No Data	Homozygous: Normal	Yes	N/A

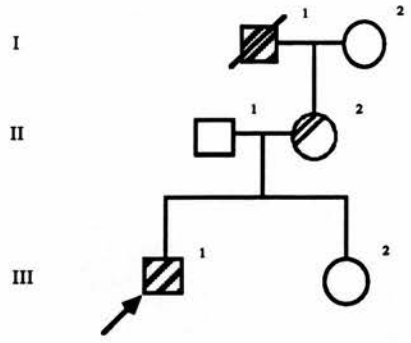
Implications and interpretations from these studies.

Although no phenotypic data was available on this family, from the pedigree I-1 and II-2 are both potential carriers. Genotypic analysis shows that the mutant gene is marked by the 0.8kb Bcl I allele. II-2 can be excluded as a carrier as she has inherited a normal 1.1kb allele from her father and a normal 1.1kb allele from her mother.

I-1 is informative for the Bcl I polymorphism and although her carrier status has not been established, she could be offered prenatal diagnosis with confirmation of an affected pregnancy be fetal blood sampling.

Family No. 44

Pedigree.



Pedigree and Phenotype Data.

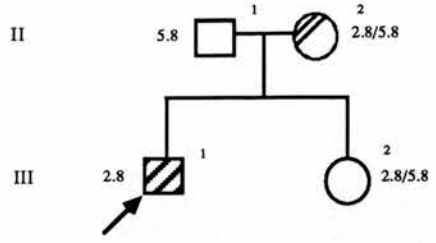
Gen. No	Sex	Carrier status from: Pedigree
II-1	M	
II-2	F	Obligate carrier
III-1	M	Propositus
III-2	F	Potential carrier

Genotype Data.

Gen. No	Sex	Bcl I	Xba I	Bgl I	Bgl II	Carrier status from Genotype
II-1	M	0.8	4.8	5.0	5.8	
II-2	F	0.8/0.8	4.8/4.8	5.0/5.0	2.8/5.8	N/A - Obligate carrier
III-1	M	0.8	4.8	5.0	2.8	Propositus
III-2	F	0.8/0.8	4.8/4.8	5.0/5.0	2.8/5.8	Carrier

Family No. 44 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 4

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-2	Obligate Carrier	No Data	Informative Bgl II: Obligate carrier	N/A	Yes
III-2	Potential carrier	No Data	Informative Bgl II: Carrier	Yes	Yes

Implications and interpretations from these studies.

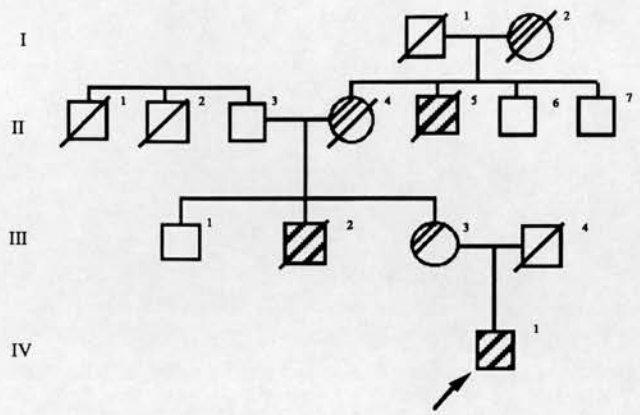
In this pedigree the haemophilic gene is associated with the 2.8kb Bgl II allele.
II-2 is an obligate carrier and informative for the linked Bgl II marker. III-2 has inherited the abnormal 2.8kb Bgl II allele from her mother and is, therefore, probably a carrier. The Bgl II RFLP could be used for prenatal diagnosis in both II-2 and III-2 if requested although with a 5% chance of error due to possible recombination.

Appendix 2.

Pedigree, Phenotypic and Genotypic Data on Families with Haemophilia B (Christmas Disease).

Family No. B1

Pedigree.



Pedigree and Phenotype Data.

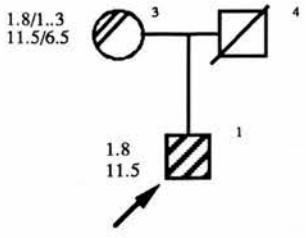
Gen. No	Sex	IX:C	IX:Ag	Carrier status from: Pedigree	Phenotype
III-3	F	1.20	0.88	Obligate carrier	Normal
IV-1	M	0.05	0.07	Propositus	

Genotypic Data.

Gen. No	Sex	Taq I	Xmn I	Carrier status from Genotype
III-3	F	1.3/1.8	6.5/11.5	N/A - Obligate carrier
IV-1	M	1.8	11.5	Propositus

Family No. B1 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members.



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 2

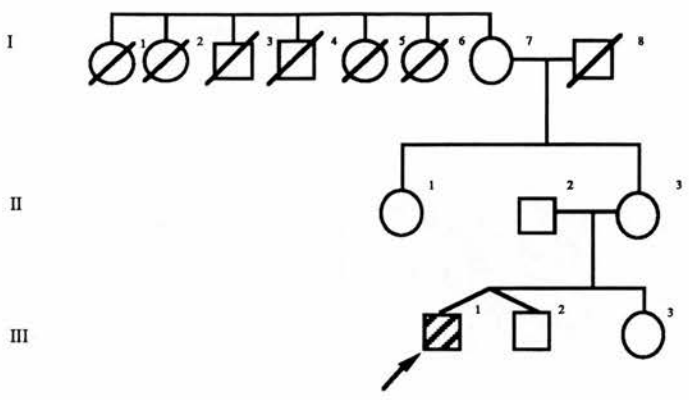
Gen. No.	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
III-3	Obligate carrier	Normal	Informative Taq I/ Xmn I: Obligate carrier	N/A	Yes

Implications and interpretations from these studies:

III-3 is an obligate carrier of haemophilia B having a single affected son and a family history. She is informative for both the Taq I and Xmn I polymorphisms and either of these could be used for prenatal diagnosis if requested.

Family No. B2

Pedigree.



Pedigree and Phenotype Data.

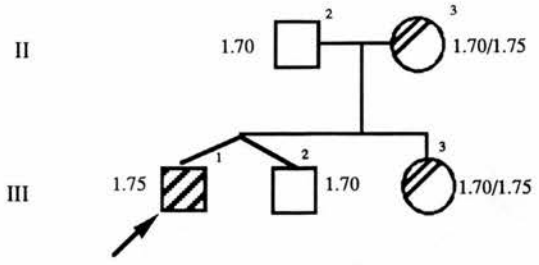
Gen. No	Sex	IX:C	IX:Ag	Carrier status from: Pedigree	Phenotype
II-2	M	1.20	1.30		
II-3	F	0.40	0.30	Potential carrier	Carrier
III-1	M	0.15	0.12	Propositus	
III-2	M	0.94	0.56		
III-3	F	0.32	0.40	Potential carrier	Carrier

Genotypic Data.

Gen. No	Sex	Taq I	Xmn I	Dde I	Carrier status from Genotype
II-2	M	1.8	11.5	1.70	
II-3	F	1.8/1.8	11.5/11.5	1.70/1.75	Potential carrier
III-1	M	1.8	11.5	1.75	Propositus
III-2	M	1.8	11.5	1.70	
III-3	F	1.8/1.8	11.5/11.5	1.70/1.75	Carrier

Family No. B2 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members.



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 5

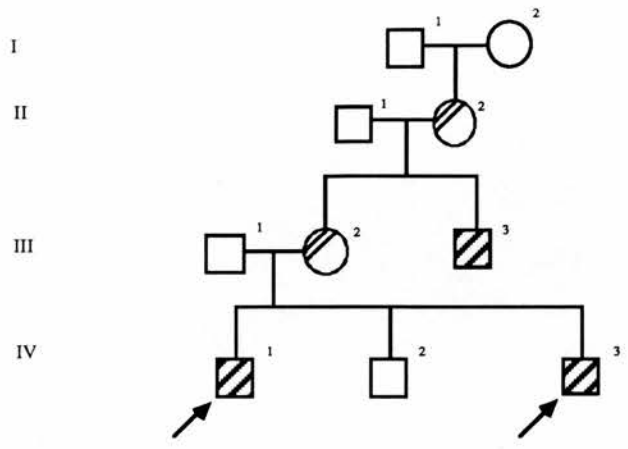
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic data	Applications of genotypic data to: CD	AND
II-3	Potential carrier	Carrier	Heterozygous Dde I: Carrier	N/A	Yes
III-3	Potential carrier	Carrier	Heterozygous Dde I: Carrier	Yes	Yes

Implications and interpretations from these studies:

In this pedigree the haemophilic gene is marked by the 1.75kb allele of the Dde I polymorphism. II-3 and III-3 are potential carriers of haemophilia B and both have abnormal coagulation phenotypes. Assuming II-3 is a carrier then III-3 has inherited the haemophilic haplotype (supported by the abnormal phenotype). Both II-3 and III-3 are informative for the Dde I polymorphism and could be offered prenatal diagnosis if requested. This family illustrates the value of a combined approach to carrier detection in apparently sporadic disease utilising both phenotypic and genotypic data. III-1 and III-2 are non-identical twins.

Family No. B3

Pedigree.



Pedigree and Phenotype Data.

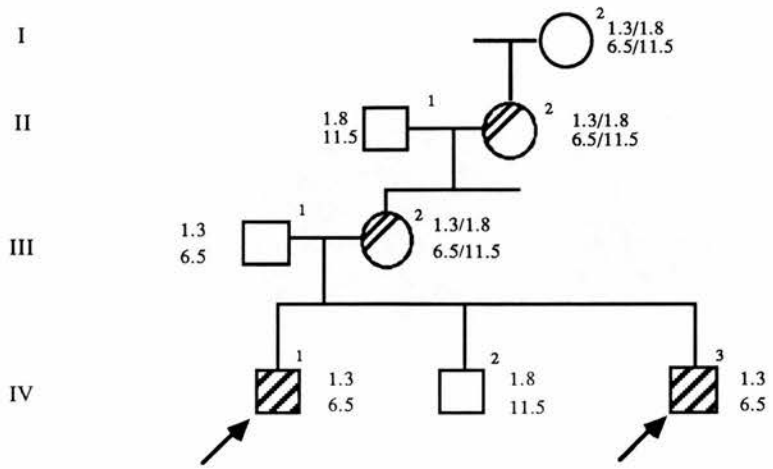
Gen. No	Sex	IX:C	IX:Ag	Carrier status from: Pedigree	Phenotype
I-2	F	No data		Potential carrier	No data
II-1	M	1.00	-		
II-2	F	0.25	-	Obligate carrier	Carrier
III-1	M	1.00	1.00		
III-2	F	0.48	0.60	Obligate carrier	Carrier
IV-1	M	0.06	0.07	Propositus	
IV-2	M	1.00	0.80		
IV-3	M	0.06	0.07	Propositus	

Genotypic Data.

Gen. No	Sex	Taq I	Xmn I	Carrier status from Genotype
I-2	F	1.3/1.8	6.5/11.5	Potential carrier
II-1	M	1.8	11.5	
II-2	F	1.3/1.8	6.5/11.5	N/A - Obligate carrier
III-1	M	1.3	11.5	
III-2	F	1.3/1.8	6.5/11.5	N/A - Obligate carrier
IV-1	M	1.3	6.5	Propositus
IV-2	M	1.8	11.5	
IV-3	M	1.3	6.5	Propositus

Family No. B3 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members.



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 8

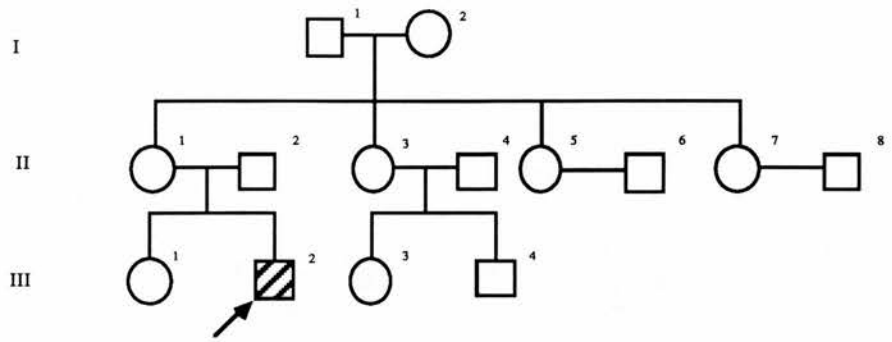
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
I-2	Potential Carrier	Carrier	Heterozygous Taq I/Xmn I: Potential carrier	N/A	Yes
II-2	Obligate carrier	Carrier	Informative Taq I/Xmn I: Obligate carrier	N/A	Yes
III-2	Obligate carrier	Carrier	Informative Taq I/Xmn I: Obligate carrier	N/A	Yes

Implications and interpretations from these studies:

In this pedigree the haemophilic gene is marked by the [1.3/6.5] Taq I/Xmn I haplotype. II-2 and III-2 are both obligate carriers, have abnormal coagulation phenotypes and are informative for both the Taq I and Xmn I polymorphisms either of which could be used in prenatal diagnosis. The carrier status of I-2 cannot be established from the available data.

Family No. B4

Pedigree.



Pedigree and Phenotype Data.

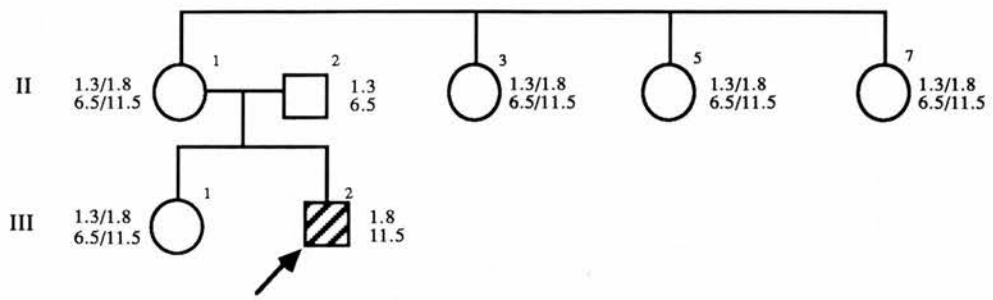
Gen. No	Sex	IX:C	Carrier status from: Pedigree	Phenotype
II-1	F	0.50	Potential carrier	Carrier
II-2	M	-		
II-3	F	0.80	Potential carrier	Normal
II-5	F	-	Potential carrier	
II-7	F	-	Potential carrier	
III-1	F	-	Potential carrier	
III-2	M	0.15		Propositus

Genotypic Data.

Gen. No	Sex	Taq I	Xmn I	Carrier status from Genotype
II-1	F	1.3/1.8	6.5/11.5	Potential carrier
II-2	M	1.3	6.5	
II-3	F	1.3/1.8	6.5/11.5	Potential carrier
II-5	F	1.3/1.8	6.5/11.5	Potential carrier
II-7	F	1.3/1.8	6.5/11.5	Potential carrier
III-1	F	1.3/1.8	6.5/11.5	Potential carrier
III-2	M	1.8	11.5	Propositus

Family No. B4 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members.



Summary of pedigree, phenotypic and genotypic data in obligate and potential carriers.

Number of Family members studied: 7

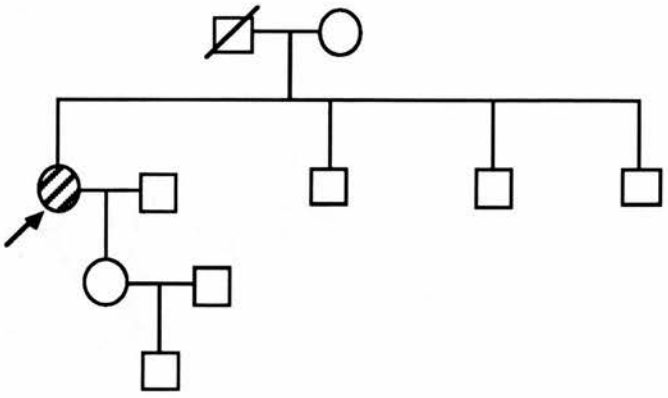
Gen. No	Status from Pedigree	Status from Phenotype	Genotypic Data/Status	Applications of genotypic data to: CD	AND
II-1	Potential carrier	Normal	Heterozygous Taq I/Xmn I: Potential carrier	No	Yes
II-3	Potential carrier	Normal	Heterozygous Taq I/Xmn I: Potential carrier	No	Yes
II-5	Potential carrier	Normal	Heterozygous Taq I/Xmn I: Potential carrier	No	Yes
II-7	Potential carrier	Normal	Heterozygous Taq I/Xmn I: Potential carrier	No	Yes
III-1	Potential carrier	Normal	Heterozygous Taq I/Xmn I: Potential carrier	No	Yes

Implications and interpretations from these studies:

In this kindred the haemophilic haplotype [1.8/11.5] is found in all 5 potential carriers (II-1, II-3, II-5, II-7 and III-1). Unfortunately, they all have a normal coagulation phenotype and it is impossible to accurately assign carrier status. Ideally the kindred specific defect should be characterised and used in carrier detection.

Family No. B5

Pedigree.

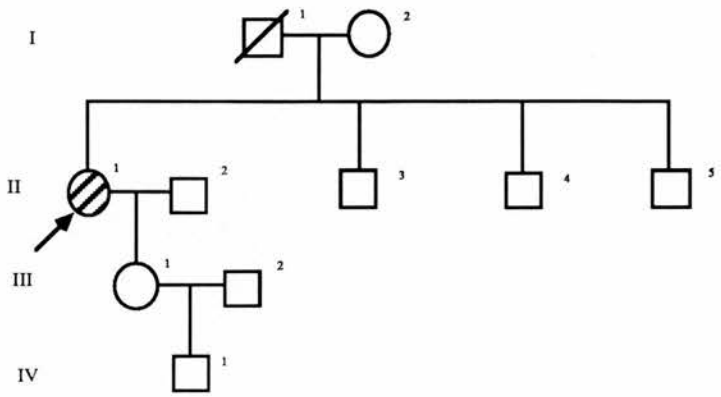


Pedigree and Phenotype Data.

Gen. No	Sex	IX:C	IX:Ag
II-1	F	0.16	0.12

Family No. B5 (continued).

Abbreviated Pedigree showing informative polymorphisms in investigated members.



Number of Family members studied: 1

Implications and interpretations from these studies:

This family is discussed in detail on at the end of Chapter 3.

II-1 was found to have low levels of both IX:C and IX:Ag although no family history of Christmas disease. Further investigations demonstrates an unusual chromosomal karyotype - XO/XX^{inv}. Amplification and sequencing of the FIX gene demonstrated a single point mutation at codon 76 resulting in the substitution of a glycine by a valine. Coagulation testing and karyotype analysis in III-1 were entirely normal.

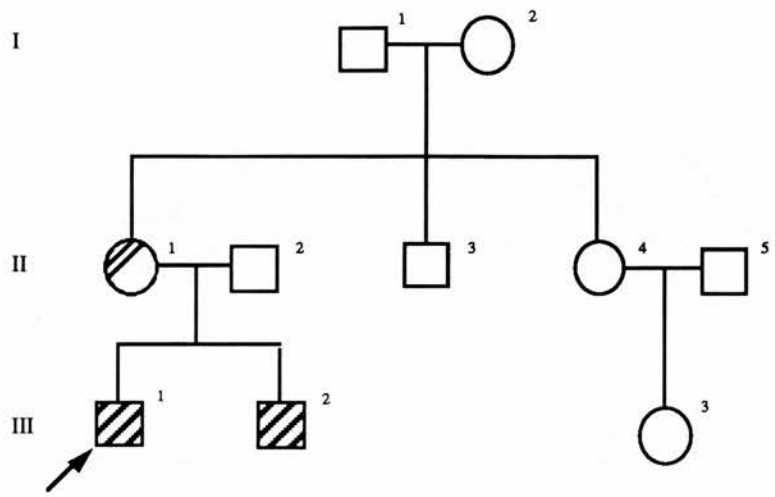
Appendix 3.

Pedigree, Phenotypic and Genotypic Data on Non-Haemophilic Families.

Family No. C1

Diagnosis: X-linked Adrenoleukodystrophy.

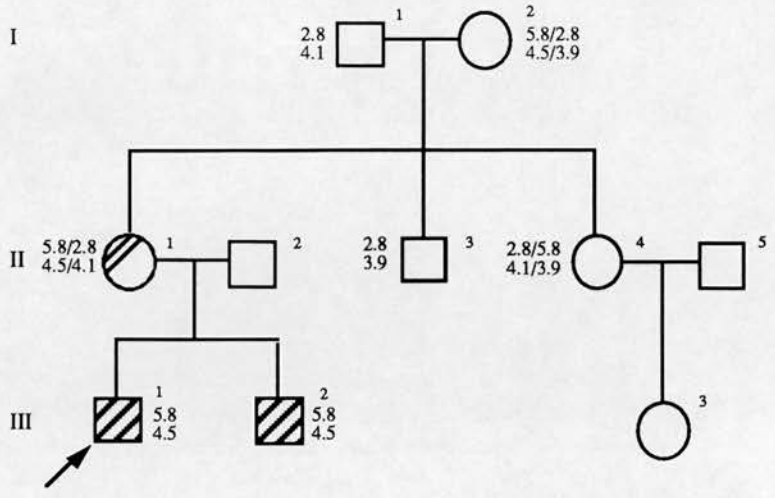
Pedigree.



Results from genotypic investigations.

Gen. No	Sex	Bcl I	Xba I	Bgl I	Bgl II	Taq I
I-1	M	0.8	4.8	5.0	2.8	4.1
I-2	F	0.8/0.8	4.8/4.8	5.0/5.0	5.8/2.8	4.5/3.9
II-1	F	0.8/0.8	4.8/4.8	5.0/5.0	5.8/2.8	4.5/4.1
II-3	M	0.8	4.8	5.0	2.8	3.9
II-4	F	0.8/0.8	4.8/4.8	5.0/5.0	2.8/5.8	4.1/3.9
III-1	M	0.8	4.8	5.0	5.8	4.5
III-4	M	0.8	4.8	5.0	5.8	4.5

Pedigree No. C1 (continued)



Summary of the pedigree, phenotypic and genotypic data.

Number of Family members studied: 7

Gen. No	Status from Pedigree	Status from Phenotype	Genotypic data
I-2	Potential carrier	No data available	Heterozygous Bgl II/Taq I: Potential carrier
II-1	Obligate carrier	Carrier	Heterozygous Bgl II/Taq I: Obligate carrier
II-4	Potential carrier	No data available	Heterozygous Bgl II/Taq I ?Normal

Implications and interpretations from these studies:

In this family a single boy (III-1) was found to be suffering from X-linked adrenoleukodystrophy. Subsequent investigations on his brother (III-2) showed that he was also affected. Studies on their mother (II-1) showed abnormal long chain fatty acids consistent with carriership of the disease. The finding of two affected individuals with the same mother (II-1) establishes her as an obligate carrier. As the gene maps close to that of the FVIII locus, the use of the FVIII gene probes allows carrier detection studies using RFLP's (See Chapter 2). Genotypic analysis of this family shows them to be non-informative for the three intragenic RFLP's but informative for the linked Bgl II and Taq I polymorphisms. The abnormal haplotype [4.5/5.8] is derived from I-2.
II-4 shows a recombination between the Bgl II (Probe DX13 (DXS15)) and Taq I (Probe St14 (DXS52)) loci. However, despite this recombination, the DXS52 loci, which is thought to be closest to the ALD locus reduces the risk that II-4 is a carrier.
Correct paternity is assumed in this family but was not confirmed. The DXS52 and DXS15 loci are physically close to each other and, therefore, recombination is unlikely.